Impaired Regulatory T-Cell Response and Enhanced Atherosclerosis in the Absence of Inducible Costimulatory Molecule

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Background—T-cell–mediated immunity contributes to the pathogenesis of atherosclerosis, but little is known about how these responses are regulated. We explored the influence of the inducible costimulatory molecule (ICOS) on atherosclerosis and associated immune responses.

Methods and Results—Bone marrow chimeras were generated by transplanting ICOS-deficient or wild-type bone marrow into irradiated atherosclerosis-prone, LDR receptor–deficient mice, and the chimeric mice were fed a high-cholesterol diet for 8 weeks. Compared with controls, mice transplanted with ICOS-deficient marrow had a 43% increase in the atherosclerotic burden, and importantly, their lesions had a 3-fold increase in CD4+ T cells, as well as increased macrophage, smooth muscle cell, and collagen content. CD4+ T cells from ICOS-deficient chimeras proliferated more and secreted more interferon-γ and tumor necrosis factor-α than T cells from control mice, which suggests a lack of regulation. FoxP3+ regulatory T cells (Treg) were found to constitutively express high ICOS levels, which suggests a role for ICOS in Treg function. ICOS-deficient mice had decreased numbers of FoxP3+ Treg and impaired in vitro Treg suppressive function compared with control mice.

Conclusions—ICOS has a key role in regulation of atherosclerosis, through its effect on regulatory T-cell responses. (Circulation. 2006;114:2047-2055.)

Key Words: atherosclerosis ■ immune systems ■ inflammation ■ leukocytes ■ cells ■ lymphocytes

Atherosclerosis is characterized by the accumulation of lipids and chronic inflammatory cells in arterial vessel walls.1 Although there is ample evidence that immune responses contribute to the pathogenesis of this common disease, there are many unanswered questions about how those immune responses are regulated and how they may be targeted for therapeutic intervention.2 CD4+ T-helper (Th) cells are evident in the atherosclerotic plaque and have been shown to be important in the development of atherosclerosis.3,4 Immune responses mediated by CD4+ Th1 cells specific for plaque antigens such as oxidized LDL (oxLDL) or heat shock protein 60/65 may have an important role in the propagation of the inflammatory process.5 CD4+ T-cell responses to antigen are modulated by costimulatory signals, delivered by B7 family molecules on antigen-presenting cells that bind to CD28 family molecules on the T cells. We have shown that the absence of B7-1 and B7-2 significantly reduces the progression of atherosclerosis.6 Inducible costimulatory molecule (ICOS) and its ligand (ICOS ligand) are CD28 and B7 family members, respectively, that also have an important role in modulating T-cell activation. ICOS is expressed on activated T cells after antigen stimulation7 and may be important in secondary activation of differentiated T cells. ICOS appears to support T-cell proliferation and Th2 differentiation.8-10 In addition, ICOS has been implicated in regulatory T-cell (Treg) function.11-12 ICOS-deficient mice are more susceptible to experimental autoimmune encephalitis.7 ICOS has also been shown to be important in antibody isotype switching.7,13,14

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Recent evidence suggests that the local balance between effector and natural regulatory T cells influences the development of atherosclerosis in animal models.15,16 Furthermore, Th1 proinflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-18, and IL-12 may have a key role in promoting atherogenesis,17,18 whereas T-cell responsiveness to transforming growth factor-β (TGF-β) has been shown to...
limit atherosclerosis. In addition, natural antibodies specific for oxidized phospholipids may have a protective effect on the development of atherosclerotic lesions. Because ICOS is reported to influence effector T-cell, antibody, and regulatory T-cell responses, we hypothesized that the ICOS pathway regulates immune responses to plaque antigens and thereby influences development of arterial disease. To test this hypothesis, we used a bone marrow chimeric approach to examine the influence of ICOS deficiency on atherosclerosis in LDL receptor (LDLR)–deficient mice. Our findings show that ICOS deficiency in bone marrow–derived cells results in enhanced atherosclerosis and associated immune responses due to the requirement of ICOS for development and function of atheroprotective natural Treg.

**Methods**

**Mice**

LDLR/−/− mice, backcrossed 10 times onto a C57BL/6 background, and wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Me). ICOS/−/− mice were derived as described elsewhere and were backcrossed 10 generations onto a C57BL/6 background. All mice were housed and bred in accordance with the institutional guidelines of Brigham and Women’s Hospital and Harvard Medical School.

**Bone Marrow Transplantation Protocol**

At 8 weeks of age, LDLR/−/− mice were lethally irradiated with a cesium source (total dose 1300 rad, split into 2 doses 4 hours apart). Donor bone marrow cells were injected after the second irradiation. Eight-week-old ICOS/−/− or wild-type donor mice were euthanized by CO2 asphyxiation, and femurs, tibias, and humeri were harvested by CO2 asphyxiation, and femurs, tibias, and humeri were harvested by Cs137 source (total dose 1300 rad, split into 2 doses 4 hours apart). Bone marrow cells were extracted, and 20 million cells were injected via tail vein into sex-matched irradiated LDLR/−/− recipient mice. Recipient mice received normal chow and water containing sulfamethoxazole (4.14 mg/mL) and trimethoprim (0.83 mg/mL) for 4 weeks.

**Induction of Atherosclerosis**

Four weeks after the transplantation, the mice were begun on a semipurified cholate-free diet (No. D12108; Research Diets Inc, New Brunswick, NJ) containing 40% kcal lipid, 1.25% cholesterol22 and 0.83% cholesterol-enriched diet. Total serum cholesterol levels, triglycerides, and plasma lipoproteins were analyzed by an online dual-enzymatic method for simultaneous quantification of cholesterol and triglycerides, by high-performance liquid chromatography at Skylight Biotech Inc (Akita, Japan) according to the procedure described by Usui et al25 and were expressed in milligrams per deciliter (mg/dL).

**Serum Cholesterol Analysis**

Overnight fasting serum samples were collected after 8 weeks of cholesterol-enriched diet. Total serum cholesterol levels, triglycerides, and plasma lipoproteins were analyzed by an online dual-enzymatic method for simultaneous quantification of cholesterol and triglycerides by high-performance liquid chromatography at Skylight Biotech Inc (Akita, Japan) according to the procedure described by Usui et al25 and were expressed in milligrams per deciliter (mg/dL).

**Aortic Atherosclerotic Lesion Analysis**

The aortic sinus was analyzed as described previously. Alternate 5-μm cryosections of ~200 μm of the aortic sinus were prepared. From the area in which 5 aortic valve cusps are clearly seen, alternate sections were collected for quantification and stained with oil red O according to the method of Paigen et al. Section images were captured digitally (SV Micro, Zeiss, Jena, Germany) and quantified with Image-Oro Plus software (Media Cybernetics, Silver Spring, Md). Plaque lesion area and percent of the total cross-sectional vessel wall area were quantified by an independent operator, blinded to the experimental protocol, and the results were expressed as the average of 6 sections per mouse.

**Immunohistochemistry**

Serial cryostat sections of aortic sinus adjacent to the oil red O–stained sections were stained with the respective molecular-specific antibodies, as described previously. Antibodies included rat anti-mouse Mc-3 for macrophage identification (1:1000, BD Pharmingen, San Diego, Calif) and anti-CD4+ for T cells (1:120, BD Pharmingen). For mouse smooth muscle cell actin staining, primary antibody (fluorescein isothiocyanate [FITC]–conjugated α-actin, 1:500, Sigma, St Louis, Mo) was applied, followed by anti-FITC biotin-conjugated secondary antibody (1:400, Sigma). Proliferating cells were stained by rabbit antinuclear antigen Ki67 antibody (Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom). For FoxP3 staining, we used a goat anti-mouse/rat FoxP3 antibody FJK-16s (ebiScience, San Diego, Calif).

Quantitative analysis of lesional content of macrophages, smooth muscle cells, and collagen was determined by computer-assisted image analysis6 and expressed as percentage of intimal area to normalize for overall differences between the study groups. Quantification of CD4, Ki67, and FoxP3 staining was done by counting individual positively stained lesional cells, which are easily resolved, in the aortic sinus sections.

**Polymerase Chain Reaction Analysis for Engraftment**

To assess engraftment, the presence of the LDLR DNA gene product (383 bp) was analyzed by polymerase chain reaction (PCR) in peripheral blood leukocytes (PBLS) sampled from the recipient mice 4 weeks after bone marrow transplantation (before initiation of high-cholesterol diet) and in CD4+ splenocytes obtained when mice were euthanized as previously described with a GFX genomic DNA purification kit (GE Healthcare Bio-Sciences, Piscataway, NJ).

**Serum Immunoglobulin Analysis**

IgM, IgG1, IgG2c, and IgG3 antibodies specific for malondialdehyde LDL and copper-oxidized (Cu-Ox) LDL were detected by isotype-specific ELISA as described previously. IgG2c was detected with an IgG2a/c-specific reagent that may underestimate IgG2c levels. We also determined titers in serum of IgM autoantibodies to oxLDL with the T15/E06 idiotype as described previously.

**Ex Vivo Assays of CD4+ Proliferation and Cytokine Secretion**

After 8 weeks of diet, splenic CD4+ T cells were isolated by anti-CD4 microbeads (Miltenyi Biotec, Auburn, Calif). The cells were stimulated in 96-well cultures (2.5×10^5/well) with plate-bound anti-CD3ε (145-2C11, BD Pharmingen) or human copper-oxidized LDL (Biomedical Technologies Inc, Stoughton, Mass) plus irradiated spleen cells (2.5×10^6/well). Ovalbumin (10 µg/mL) and medium alone were used as controls. Culture supernatants were removed at 48 hours and analyzed by flow cytometry–based cytokine bead assays (BD Pharmingen) of culture supernatants for IFN-γ, IL-2, tumor necrosis factor (TNF)-α, IL-4, IL-5, and IL-10. Cultures were assayed for proliferation after 64 hours by uptake of [3H]thymidine (1 µCi/well), added 16 hours before harvest. Data are expressed as mean proliferation indices of triplicates calculated from the ratios of incorporated radioactive counts per minute in the presence or absence of antigen.
Real-Time PCR
CD4⁺ spleen cells stimulated with anti-CD3 were analyzed for cytokine production by TaqMan real-time PCR (RT-PCR). Total RNA was isolated from 5×10⁶ million purified T cells, reverse transcribed, and analyzed by quantitative RT-PCR with SYBR green dye. All real-time reactions were performed on the iCycler iQ real-time PCR detection system (Bio-Rad), and analysis was done with the accompanying software. The presence of single amplicons resulting from RT-PCR was verified by dissociation curve analysis. Levels of specific gene expression in the samples are presented relative to endogenous levels of β-actin housekeeping gene expression in the same sample.

Flow Cytometry
Lymphocytes were stained with BD Biosciences phycoerythrin (PE)- or allophycocyanin-conjugated anti-CD25 (PC61), PE- or FITC- or PE-Cy5-conjugated anti-CD4 (L3T4), FITC-conjugated anti-CD3, and PE-conjugated anti-ICOS (7E.17G9). For intracellular staining of FoxP3, a FoxP3-allophycocyanin antibody (FJK-16s) and Foxp3 staining buffer set from eBioscience were used according to the manufacturer’s recommended protocol. All samples were analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences) as described previously.

Regulatory T-Cell Isolation and Functional Assays
Tregs were isolated from spleen cell suspensions by the CD4⁺ regulatory T cell isolation kit (Miltenyi Biotec), which depletes Tregs were isolated from spleen cell suspensions by the CD4⁺ regulatory T cell isolation kit (Miltenyi Biotec), which depletes Tregs were isolated from spleen cell suspensions by the CD4⁺ regulatory T cell isolation kit (Miltenyi Biotec), which depletes Tregs were isolated from spleen cell suspensions by the CD4⁺ regulatory T cell isolation kit (Miltenyi Biotec), which depletes Tregs were isolated from spleen cell suspensions by the CD4⁺ regulatory T cell isolation kit (Miltenyi Biotec), which depletes CD4⁺ T cells followed by positive selection of CD4⁺CD25⁺ cells. The purity of the isolated CD4⁺CD25⁺ cells and CD4⁺CD25⁻ populations was >95% by fluorescence-activated cell sorter analysis. CD4⁺CD25⁺ responder cells (5×10⁶/well) were cultured in 96-well plates (0.2 mL) with soluble anti-CD3 (1 μg/mL) plus irradiated spleen cells (5×10⁶/well) and increasing numbers of CD4⁺CD25⁺ regulatory T cells (responder to suppressor ratio of 1:16 to 1:1) for 72 hours. Cultures were pulsed with [3H]thymidine for the last 16 hours of culture. Culture supernatants were removed at 48 hours and analyzed for cytokines, as described above. Anti-TGF-β–blocking antibody (R&D Systems Inc, Minneapolis, Minn) was added to some suppression assays at 25 μg/mL.

Statistical Analysis
All statistical analyses were performed with Prism software (GraphPad Software, Inc, San Diego, Calif). Differences between groups of mice were analyzed by the Student t test or the Mann-Whitney test (for data that did not pass the Kolmogorov-Smirnov normality test), and data are expressed as mean±SEM.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Bone Marrow Engraftment, Weights, and Serum Lipids
To confirm engraftment, mice were bled at 4 weeks after transplantation, and PBLs were counted. There was no difference in total cell number between the groups (7.7±1.2×10⁶/mL versus 9.1±0.8×10⁶/mL in wild-type and ICOS-null cell recipients, respectively, P=0.35). To further assess engraftment, we looked for the presence of wild-type
and mutant LDLR genes by PCR in PBLs and splenocytes of the recipient mice. The wild-type product was equally abundant in recipients of ICOS-null or wild-type marrow (Figure 1A). Importantly, there was also no ICOS protein expression on PBLs sampled at 4 weeks or on CD4+/H11001 splenocytes obtained at the time of euthanasia from recipients of ICOS+/H11002 bone marrow (Figure 1B).

There was no significant difference in male or female weights between the groups (Table 1). The analysis of total serum cholesterol levels, as determined by enzymatic assay, revealed no statistically significant differences between the 2 groups (Table 2).

### TABLE 1. Mean Weights of Mice According to Sex

<table>
<thead>
<tr>
<th>Group</th>
<th>12 Weeks (Diet Initiation)</th>
<th>20 Weeks (Euthanasia)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT→LDLR+/−</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23.2±0.3*</td>
<td>26.3±0.9</td>
</tr>
<tr>
<td>Female</td>
<td>20.4±0.7</td>
<td>21.8±0.8</td>
</tr>
<tr>
<td><strong>ICOS−/−→LDLR−/−</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24.4±0.3</td>
<td>27.4±0.6</td>
</tr>
<tr>
<td>Female</td>
<td>18.9±0.2</td>
<td>20.9±0.6</td>
</tr>
</tbody>
</table>

*P<0.05 when comparing mice of the same sex between the 2 groups.

### TABLE 2. Serum Lipids and Lipoproteins at Time of Euthanasia

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Total cholesterol, mg/dL</th>
<th>LDL cholesterol, mg/dL</th>
<th>HDL cholesterol, mg/dL</th>
<th>Total triglycerides, mg/dL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT→LDLR+/−</td>
<td>787.0±26.1</td>
<td>383.4±9.2</td>
<td>88.6±4.9</td>
<td>115.2±17.7</td>
<td>0.4</td>
</tr>
<tr>
<td>ICOS−/−→LDLR−/−</td>
<td>862.5±77.0</td>
<td>405.2±38.4</td>
<td>80.2±6.1</td>
<td>119.5±22.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Quantitative and Phenotypic Differences in Atherosclerotic Lesions in ICOS-Deficient and Control Mice**

After 8 weeks of high-cholesterol diet, mice transplanted with ICOS−/− cells had significantly more atherosclerosis in the aortic sinus than control mice transplanted with wild-type cells (Figure 2). In mice transplanted with ICOS−/− cells, average lesion area was increased from 309,000±42,000 μm² (n=10) to 474,000±37,000 μm² (n=15; P<0.05; Figure 2C). Fractional lesion area was increased from 37.4±2.2% to 53.6±2.2% (P<0.0001), a 43% increase in the atherosclerotic burden (Figure 2D). The increase in atherosclerosis in recipients of ICOS−/− cells was significant when atherosclerosis was evaluated according to sex. For wild-type versus ICOS−/− males, the respective fractional areas were 34.5±1.8% (n=6) versus 50.2±3.1% (n=7; P<0.005). For wild-type versus ICOS−/−
females, the respective fractional areas were 41.7±4.3% (n=4) versus 56.6±2.8% (n=8; P<0.01). We found minimal and unquantifiable atherosclerotic lesions in the aortic arch and descending aorta, consistent with previous reports of irradiated LDLR<sup>−/−</sup> bone marrow recipients.29 After 8 weeks of proatherogenic diet, there was a 3-fold increase in CD4<sup>+</sup> T cells in the intima of mice transplanted with ICOS<sup>−/−</sup> cells (Figures 3A, 3B, and 3K). In addition, there was increased smooth muscle cell content (Figures 3E, 3F, and 3M) and collagen deposition (Figures 3G, 3H, and 3N) in these mice. These differences between wild-type and ICOS<sup>−/−</sup> groups were significant when both sexes were evaluated together and when each sex was evaluated separately. There also were more macrophages in the ICOS<sup>−/−</sup> group than the control group (Figures 3C, 3D, and 3L), but the difference was not statistically significant. The present immunohistochemical data suggest that mice transplanted with ICOS<sup>−/−</sup> cells have more active atherosclerotic lesions with more T-cell infiltration; more local proliferation of T cells, macrophages, and/or smooth muscle cells; and more synthesis of extracellular matrix.

Enhanced Immunologic Responses in ICOS-Deficient Mice

To examine whether ICOS deficiency altered T-cell function, we isolated CD4<sup>+</sup> T cells from mice that received ICOS<sup>−/−</sup> or wild-type bone marrow and compared their responses to polyclonal and antigen-specific stimuli. CD4<sup>+</sup> T cells isolated from mice transplanted with ICOS<sup>−/−</sup> cells had a significantly greater proliferative response to anti-CD3 or oxLDL than control mice (Figure 4A). Although the magnitude of the oxLDL-specific response was low, the differences were consistent and statistically significant when T cells were stimulated with 10 μg/mL oxLDL (Figure 4B).

CD4<sup>+</sup> T cells isolated from mice transplanted with ICOS<sup>−/−</sup> bone marrow secreted more Th1 cytokines (IL-2, IFN-γ, and Ki67-specific antibody, in mice transplanted with ICOS<sup>−/−</sup> cells compared with control mice (Figures 3I, 3J, and 3O). Taken together, these immunohistochemical data suggest that mice transplanted with ICOS<sup>−/−</sup> cells have more active atherosclerotic lesions with more T-cell infiltration; more local proliferation of T cells, macrophages, and/or smooth muscle cells; and more synthesis of extracellular matrix.

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CD4<sup>+</sup> T cells isolated from mice transplanted with ICOS<sup>−/−</sup> bone marrow secreted more Th1 cytokines (IL-2, IFN-γ, and
TNF-α) and more Th2 cytokines (IL-10, IL-4, and IL-5) on stimulation with anti-CD3 (Figures 4C and 4D) than CD4+ T cells isolated from controls. In contrast, the CD4+ T cells from ICOS−/− bone marrow recipients produced significantly less anti-inflammatory cytokine TGF-β than CD4+ T from control mice (Figure 4E). Quantitative RT-PCR analysis of the cytokine mRNA expression of the stimulated CD4+ T cells was concordant with the cytokine protein assays, with the exception of IL-10, which did not differ significantly between the experimental groups (Figures 4F through 4H).

We found a significant increase in serum monocyte chemotactic protein-1 and a trend for increased TNF-α in mice transplanted with ICOS−/− cells compared with controls (Figure 4I). There were no significant differences in IFN-γ, IL-10, IL-12, and IL-6. These results are consistent with an enhanced systemic inflammatory response in the mice with ICOS-deficient marrow.

Antibody responses to oxLDL may modulate atherosclerotic disease, and ICOS may influence production of these antibodies.20,21 We therefore measured titers of serum antibodies specific for different forms of oxLDL (Figure 5). There were statistically significant differences in IFN-γ, IL-10, IL-12, and IL-6. These results are consistent with an enhanced systemic inflammatory response in the mice with ICOS-deficient marrow.

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Relationship Between ICOS and Regulatory T-Cell Numbers and Function

In light of published data that suggest that ICOS may be important in Treg function and the present data indicating enhanced immune responses in the absence of ICOS, we evaluated the relationship between ICOS and Treg. Using 2 specific molecular markers of these cells, CD25 and intracellular FoxP3, we found that the frequency of ICOS-expressing cells was 3-fold higher in the CD4+ Treg subset than in the CD4+ T non-Treg subset (Figure 6A). These data suggest that ICOS has a role in the development or function of this T-cell subset. We therefore examined Treg counts and function in ICOS−/− mice (unirradiated, LDLR−/−). We found a 30% reduction in Treg counts as evidenced by FoxP3 staining in the spleen of ICOS−/− mice on regular or high-cholesterol diet compared with control mice (Figure 6B). There was no significant difference in total spleen CD4+ T-cell counts between the groups. We also quantified Treg in LDLR−/− mice transplanted with ICOS−/− versus wild-type bone marrow on high-cholesterol diet for 8 weeks. For this analysis, we included iliac lymph nodes, which drain the atherosclerotic descending aorta. Treg counts were reduced by 40% in the iliac lymph nodes and the spleen in mice transplanted with ICOS−/− cells compared with control mice (Figure 6C). These data support the hypothesis that ICOS positively influences the development or maintenance of Treg.

The presence of ICOS on Treg, together with the increased number of T cells and enhanced lesion formation in mice with ICOS-deficient bone marrow, suggested that Treg may control the proatherogenic effector T cells and influence the inflammatory burden in the atherosclerotic plaque. We therefore looked for the presence of Tregs in the mouse atherosclerotic lesions by immunohistochemical staining for FoxP3. We found that mice transplanted with either wild-type or ICOS−/− bone marrow had

Figure 5. Serum titers of oxLDL-specific antibodies. IgG1, IgG2c, IgG3, and IgM antibodies that bind malondialdehyde (MDA)-LDL (A, B), copper-oxidized (Cu-Ox) LDL (C, D), and phosphorylcholine-specific (EO6) antibodies (E) were detected by isotype-specific ELISA. Data shown are mean±SEM; n=12 per group. RLU indicates relative light units.
readily detectable Treg in the atherosclerotic lesions (Figure 6D); however, we could not demonstrate significant quantitative differences between the groups. The total CD4+ T-cell counts in the lesions were more than 3-fold higher in the mice transplanted with ICOS−/− cells compared with control mice (Figure 3), which suggests that the Tregs that are present in the lesions of these mice are insufficient in number or function to suppress the local effector T-cell response.

To evaluate whether regulatory T-cell function, in addition to numbers, is influenced by ICOS expression, we compared the suppressive function of the Treg isolated from ICOS−/− and wild-type mice. Indeed, the ability of Treg from ICOS−/− mice to suppress proliferation of wild-type or ICOS−/− cell responders was reduced compared with Treg from wild-type mice (Figure 6E). Reduced suppression by ICOS−/− Treg was also clearly evident by analysis of interferon-γ and TNF-α in...
the supernatants of these assay cultures (Figures 6F and 6G). To address whether TGF-β has a role in Treg function in atherosclerotic mice, we used a TGF-β–blocking antibody in the suppression assay. We found that blocking TGF-β caused reduced suppression by Treg from \textit{LDLR}^{-/-} mice transplanted with wild-type bone marrow (Figure 6H). Anti-TGF-β did not cause significant effects on the already reduced suppression seen with Treg from \textit{LDLR}^{-/-} mice transplanted with wild-type \textit{ICOS}^{-/-} bone marrow.

### Discussion

In this study, we found that ICOS deficiency on bone marrow–derived cells increases the atherosclerotic burden in \textit{LDLR}^{-/-} mice. Importantly, the increase in lesions is associated with increased infiltration of CD4^{+} T cells, which indicates that plaque-based T-cell responses are more active in the absence of ICOS. In addition, the lesions in the ICOS-deficient mice had more smooth muscle cell and collagen deposition. The increased atherosclerotic burden in \textit{ICOS}^{-/-} bone marrow–recipient mice correlated with increased ex vivo T-cell responsiveness to polyclonal and antigen-specific stimuli and in monocye chemotactic protein serum levels. Taken together, these data imply that ICOS deficiency leads to an enhanced T-cell response to hypercholesterolemia and plaque antigens and increased atherosclerosis.

Similar to the present data, ICOS deficiency has been shown to enhance helper T-cell responses in models of autoimmune diseases, including experimental autoimmune encephalitis and insulin.\textsuperscript{11} Experimental evidence clearly show that ICOS is a positive costimulatory molecule for CD4^{+} T cells.\textsuperscript{7} Therefore, it is paradoxical that ICOS deficiency increases immune responses in vivo. A possible explanation is the role of ICOS in the development or function of Th2 cells, which could downregulate Th1 responses.\textsuperscript{8} However, recent studies show that ICOS is also involved in Th1 differentiation.\textsuperscript{3,12} Consistent with this, we saw an increase in both Th1 and Th2 cytokine production by CD4^{+} T cells isolated from ICOS-deficient hypercholesterolemic mice ex vivo. Therefore, we do not think a change in Th1:Th2 balance explains the effect of ICOS deficiency in the present study. The present data also do not support the idea that the effect of ICOS deficiency is due to changes in antibody responses to oxidatively modified LDL or phosphorylcholine, which are known to modulate atherosclerosis.\textsuperscript{2,26}

The best-characterized subset of regulatory T cells (Tregs) are the “natural” CD4^{+}CD25^{+} Tregs, which constitute \textasciitilde 10\% of peripheral CD4^{+} T cells.\textsuperscript{33} Most natural Tregs express the transcription factor FoxP3.\textsuperscript{34} We considered that impaired natural Treg activity may be the mechanism underlying the increased immune response and atherosclerotic burden in the setting of ICOS deficiency. Indeed, we found that ICOS plays an important role in induction or maintenance, as well as function, of natural Treg. There was a 40\% reduction in the number of Tregs in the absence of ICOS as determined by FoxP3 expression. This difference is especially significant given the ability of small numbers of Tregs to suppress T-cell responses. Furthermore, we found a reduction in Treg suppressive function in \textit{ICOS}^{-/-} mice assessed in vitro. This is consistent with recent studies showing that ICOS is crucial to Treg-dependent self-tolerance.\textsuperscript{11,12,35,36} Therefore, ICOS deficiency in the setting of hypercholesterolemia would be predicted to enhance proatherogenic T-cell responses and exacerbate lesion development, as we saw in the present study. A recent article reported that apolipoprotein E–deficient mice immunized with an ICOS-Ig fusion protein had increased early lesion development,\textsuperscript{37} but that study did not clarify whether and how the immunization altered the ICOS pathway in vivo, nor did it provide a mechanism by which the ICOS pathway might influence atherosclerosis. The present data are in accordance with a study demonstrating the importance of Treg in inhibiting development of atherosclerosis in apolipoprotein E mice.\textsuperscript{16}

Interestingly, the only cytokine that we analyzed whose production was decreased in mice receiving \textit{ICOS}^{-/-} cells versus control mice was TGF-β. In addition, an in vitro suppressive assay from these mice suggests that TGF-β plays a role in Treg function. This cytokine is a potent regulator of effector T-cell differentiation, and it inhibits the acquisition of specific Th1 or Th2 cell functions.\textsuperscript{38} Conversely, TGF-β promotes the development of FoxP3-expressing Tregs.\textsuperscript{39,40} Therefore, reduced TGF-β may contribute to the increased immune responses seen in the \textit{ICOS}^{-/-} mice in the present study. Overall, the present data support the notion that Tregs inhibit atherosclerosis through TGF-β secretion.\textsuperscript{16,19}

In summary, our results indicate that ICOS is important for the regulation of proatherogenic T-cell responses in hypercholesterolemic animals. Enhancement of Treg responses, through manipulation of the ICOS pathway, has potential as a therapeutic strategy for atherosclerotic disease.

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### Disclosures

None.

### References


16. Lee TS, Yen HC, Pan CC, Chau LY. The role of interleukin 12 in the initiation of most CD4+ T-cell responses to antigens and therefore may promote arterial disease. However, recent studies have shown that the B7-CD28 pathway is also essential for regulatory T-cell development and function and that regulatory T-cell populations may serve to suppress proatherogenic T cells. Inducible costimulatory molecule (ICOS) is a CD28 family member for which in vivo functions are incompletely understood. This study tested the hypothesis that ICOS modulates proatherogenic T-cell responses. The data indicate that a deficiency in ICOS in mouse bone marrow–derived cells results in a marked increase in T-cell responses to hypercholesterolemia, associated with more T-cell infiltrates in lesions and more lesion development. The effects of ICOS deficiency are related to a reduction in numbers and function of regulatory T cells. These data suggest that pharmacological blockade of costimulatory pathways as a potential therapeutic strategy for atherosclerosis must be refined to avoid impairment of the beneficial effects of regulatory T cells.

**CLINICAL PERSPECTIVE**

CD4+ T-lymphocyte–mediated adaptive immune responses contribute significantly to the inflammatory process that occurs in atherosclerotic lesions. Costimulatory pathways that involve B7 and CD28 family molecules are essential for the initiation of most CD4+ T-cell responses to antigens and therefore may promote arterial disease. However, recent studies have shown that the B7-CD28 pathway is also essential for regulatory T-cell development and function and that regulatory T-cell populations may serve to suppress proatherogenic T cells. Inducible costimulatory molecule (ICOS) is a CD28 family member for which in vivo functions are incompletely understood. This study tested the hypothesis that ICOS modulates proatherogenic T-cell responses. The data indicate that a deficiency in ICOS in mouse bone marrow–derived cells results in a marked increase in T-cell responses to hypercholesterolemia, associated with more T-cell infiltrates in lesions and more lesion development. The effects of ICOS deficiency are related to a reduction in numbers and function of regulatory T cells. These data suggest that pharmacological blockade of costimulatory pathways as a potential therapeutic strategy for atherosclerosis must be refined to avoid impairment of the beneficial effects of regulatory T cells.
Impaired Regulatory T-Cell Response and Enhanced Atherosclerosis in the Absence of Inducible Costimulatory Molecule

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