Interruption of the Tnfrsf4/Tnfsf4 (OX40/OX40L) Pathway Attenuates Atherogenesis in Low-Density Lipoprotein Receptor–Deficient Mice

Eva J.A van Wanrooij, Gijs H.M van Puijvelde, Paula de Vos, Hideo Yagita, Theo J.C. van Berkel, Johan Kuiper

Objective—Atherosclerosis is a chronic (auto-)inflammatory disease and T cell activation is an important factor in this process. Tnfrsf4 (OX40) and Tnfsf4 (OX40 ligand) are members of the tumor necrosis factor (TNF) and TNF receptor family and OX40/OX40L mediated signaling is important in co-activation of T cells and facilitates B–T cell interaction. In this study we assessed the role of the OX40/OX40L pathway in atherosclerosis and the effect of interruption of the OX40/OX40L pathway on lesion development.

Methods and Results—We treated low-density lipoprotein receptor-deficient (LDLr−/−) mice with an anti-OX40L antibody which lead to a 53% decrease in atherosclerotic lesion formation. Treatment resulted in inhibition of Th2 mediated isotype switching by decreasing interleukin (IL)-4 secretion and subsequent low IgG1 serum levels against oxLDL, whereas protective anti-oxLDL specific IgM titers were increased in treated mice compared with control.

Conclusions—We conclude that blocking the OX40/OX40L interaction reduced atherogenesis by inhibition of IL-4 mediated Th2 induced isotype switching and subsequent increased levels of anti-oxLDL IgM. (Arterioscler Thromb Vasc Biol. 2007;27:204-210.)

Key Words: atherosclerosis ■ OX40 ■ OX40L ■ isotypeswitch ■ IgM

Atherosclerosis is considered to be a chronic (auto-)inflammatory disease in the context of high plasma cholesterol levels, in which both innate and adaptive immune responses play a role. It is characterized by the infiltration of the arterial vasculature by leukocytes in response to both endothelial injury and elevated levels of oxidized lipoproteins. These elevated levels of oxidized lipids induce a strong humoral response, resulting in the production of specific antibodies against oxidized low-density lipoprotein (oxLDL) and malondialdehyde LDL (MDA-LDL).

T cell activation is an important step in this process and is initiated by antigen (possibly oxidized lipoproteins or heat shock proteins) recognition in combination with co-stimulatory signals provided by antigen presenting cells (APCs). The TNF receptor pathway can provide co-stimulatory signals and has been implicated in the onset and progression of atherosclerosis. Tnfrsf4 (OX40, CD134) is a less well-explored member of the TNF receptor family and is primarily expressed on activated CD4-positive and CD8-positive T cells in mice and humans. OX40L (Tnfsf4, CD134L) is expressed on a wide variety of cells including dendritic cells, B-lymphocytes, microglia, and vascular endothelial cells.

OX40 and OX40L have been implicated in T cell activation and migration into inflamed tissue, as well as in the activation of B cells and macrophages. Activated OX40-positive T cells can provide help to B cells via OX40L expressed on the B cell surface, and can subsequently induce isotype switching dependent on their cytokine production.

Interruption of OX40–OX40L interaction has been shown to ameliorate several autoimmune like diseases. The expression of OX40 by T cells during EAE and graft-versus-host disease (GVHD) is correlated with the severity of disease, increasing during disease onset, and decreasing when the clinical parameters decline. Interestingly, the OX40L gene is located within the Ath-1 QTL on chromosome 1, a region associated with aggravated atherosclerosis, indicating a role for this molecule in atherogenesis.

In the present study we examined the expression of OX40 during the formation of atherosclerotic lesions in low-density lipoprotein receptor-deficient (LDLr−/−) on a Western-type diet and determined the effect of an OX40L blocking monoclonal antibody (RM134) on atherogenesis.


Materials and Methods

Animal Experiments
Female LDLr⁻/⁻ mice, 8 weeks old, originally obtained from Jackson Laboratories and house bred, were put on a Western type diet containing 0.25% cholesterol and 15% cocoa butter 2 weeks before collar placement. Silastic collars (0.3-mm inside diameter; Dow Corning, Midland) were placed around the carotid artery to induce atherosclerosis as described previously. After collar placement mice were treated with 300 μg of anti-OX40L antibody (RM134) in 100 μL of sterile phosphate-buffered saline (PBS) intraperitoneally twice per week during 6 weeks. As a control rat IgG (Sigma) in sterile PBS was used. Cryosections from the carotid artery (5 μm) were stained with hematoxylin and eosin. Site of maximal stenosis was used for morphometric assessment using a Leica DM-RE microscope and Leica Qwin software (Leica imaging systems, Cambridge, UK). Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage-specific antigen (MOMA-2, monoclonal rat IgG2b, diluted 1:50). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100) was used as secondary antibody (1 hour, room temperature) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. Lesion collagen content was visualized by a 90-minute incubation of slides in 0.1% Sirius Red (Direct Red 80, Sigma) in saturated picric acid and subsequent rinsing in 0.01 mol/L HCl.

Serum Lipid Levels
Blood samples were collected by tail bleeding from nonfasted animals. Concentrations of serum cholesterol and triglycerides were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as a standard.

Real-Time Polymerase Chain Reaction Assays
Total RNA was isolated from spleen using the guanidium isothiocyanate (GTC) method. Purified RNA was DNase treated (DNase I, 10 U/μg of total RNA) and reverse-transcribed (RevertAid M-MuLV reverse transcriptase) according to manufacturer’s protocol. Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Foster City, Calif) using SYBR Green technology. Polymerase chain reaction primers (supplemental Table I) were designed using Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems) and validated for identical efficiencies (slope=-3.3 for a plot of the threshold cycle number (Ct) versus log ng cDNA). Acidic ribosomal phosphoprotein PO (36B4) and hypoxanthine phosphoribosyl transferase (HPRT) were used as housekeeping genes.

Flow Cytometry
Leukocytes from whole blood and spleen were isolated by density gradient centrifugation with Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) according to manufacturer’s protocol. Cell suspensions from spleen and blood were incubated with 1% normal mouse serum in PBS and stained for surface markers (0.25 μg Ab/200,000 cells) and subsequently subjected to flow cytometric analysis (fluorescence-activated-cell sorter [FACS]). Antibodies were purchased from eBiosciences. All data were acquired on a FACSCalibur and were analyzed with CELLQuest software (BD Biosciences).

Serum Antibody and Cytokine Level Determination
IgG1, IgG2A, and IgM levels against oxLDL were detected in serum using the Mouse MonoAb ID kit (Zymed Laboratories Inc, South San Francisco, Calif). OxLDL (5 μg/mL) dissolved in a NaHCO₃/Na₂CO₃ buffer (pH 9.0) was coated overnight onto a flat-bottom 96-well high binding plate (Corning, NY). Serum samples were 1:1 diluted in PBS and absorbance was detected at 405 nm. IL-5 concentration was determined using an IL-5 specific enzyme-linked immunosorbent assay (Immunosource).

Spleen and peritoneal cells from mice that received 2 weeks of Western type diet and were treated with control IgG or RM134 were cultured at 2×10⁶ cells/mL and stimulated with anti-CD3 and CD28 coated antibodies. Supernatants were collected after 24 hours and IFN-γ, IL-5, and IL-4 concentrations were determined by enzyme-linked immunosorbent assay (eBiosciences, Belgium).

Statistical Analysis
Values are expressed as mean±SEM. Two-tailed Student t test was used to compare normally distributed data between two groups of animals. Mann-Whitney test was used to not normally distributed data. P<0.05 was considered to be significant for both tests.
Expression of OX40 During Western-Type Diet Feeding

Atherosclerosis is characterized by both locally presented (within the vessel wall) and circulating antigens such as modified LDL. The spleen is therefore continuously exposed to atherogenic factors such as oxLDL, and the reaction of the spleen in response to a high-cholesterol diet used in our model can provide insight into the systemic immunologic response. We examined the mRNA expression of OX40 in the spleen of LDLr<sup>−/−</sup> mice fed a Western-type diet for 0, 3, 6, 9, and 12 weeks. A significant increase in the relative expression of OX40 was determined in the spleen of LDLr<sup>−/−</sup> mice, showing a strong positive correlation with time of diet administration (Figure 1A).

In comparison, the expression of IL-1β (IL-1β), a known pro-atherogenic cytokine, increased 2.8-fold only after 6 weeks of diet. Initial activation of naive T cells requires interaction between CD28 and CD80 or CD86 on activated protein C (APC). CD28 is already expressed on nonactivated T cells and no differential expression is observed during the initial stages of atherogenesis, but after 12 weeks a 9.1-fold reduction is observed. IL-12 regulates this process, and its expression profile resembles that of CD28 (supplemental Figure I, available online at http://atvb.ahajournals.org).

To correlate the elevated mRNA expression of OX40 in spleen to protein expression, we determined cell surface expression of OX40 during Western-type diet feeding. The expression of CD4, CD8, and OX40 in spleen and blood was determined in female LDLr<sup>−/−</sup> mice using FACS analysis at 0, 2, and 4 weeks of Western-type diet feeding. The expression is plotted as the percentage OX40<sup>+</sup> cells in the CD4<sup>+</sup> or CD8<sup>+</sup> positive population. In the spleen, a 2-fold increase was found in both CD4/OX40 double-positive and CD8/OX40 double-positive T cells after 2 and 4 weeks of Western type diet as compared with control. The induction of OX40 expression in the spleen is associated with an increase in the expression of OX40 in the circulation. Within the CD4<sup>+</sup> population, a significant increase in the OX40 positive population is observed in blood after 2 and 4 weeks of Western-type diet feeding as compared with chow fed mice. The percentage OX40 positive cells in the CD8<sup>+</sup> population shows a 5-fold increase as compared with chow fed mice on both 2 and 4 weeks of Western type diet (Figure 1B).

Attenuation of Atherogenesis by Anti-OX40L Antibody Administration

OX40-positive T cells require OX40L on the vascular endothelium to enter inflamed tissue from the circulation, and this interaction can be inhibited by the anti-OX40L antibody RM134. Furthermore, the anti-OX40L antibody can inhibit T cell activation, and subsequently reduce their help to B cells.

To investigate the effect of specific blockade of OX40L on atherogenesis, we used the anti-OX40L antibody in LDLr<sup>−/−</sup> mice. After 2 weeks of Western-type diet, female LDLr<sup>−/−</sup> mice were equipped with pericardiot collars and subsequently treated twice per week with 300 µg of anti-OX40L for a period of 6 weeks. Figure 2 shows representative sections of the aortic root of control IgG treated mice (D) and anti OX40L treated mice (E) are shown. A marked and significant decrease was seen for carotid plaque area. Error bars represent SEM, n=10 per group (P<0.05, Mann Whitney test). Representative photomicrographs of oil red O-stained cross-sections of the aortic root of control IgG treated mice (D) and anti OX40L treated mice (E) are shown. A significant reduction in plaque size was found as compared with control (P<0.01, n=10 per group) (F).

In addition, we quantified the lesion size in both 2 and 4 weeks of Western type diet feeding. The percentage OX40 positive cells in the CD8<sup>+</sup> population shows a 5-fold increase as compared with chow fed mice on both 2 and 4 weeks of Western type diet (Figure 1B).
significant difference in relative macrophage or collagen content was observed (supplemental Figure III).

Expression of OX40 After Anti-OX40L Treatment
After euthanizing the animals we determined the mRNA expression of OX40. A significant 45% decrease in the spleen of α-OX40L–treated mice (6 weeks) is expressed relative to 36B4 and HPRT, and subsequently related to the expression in control IgG treated mice. White bars represent control mice (n=10), black bars represent anti-OX40L–treated mice (n=10). A significant decrease could be observed in OX40 expression after anti-OX40L treatment. Results in B panels represent the percentage of CD4^+ OX40^+ or CD8^+ OX40^+ positive cells ± SEM from 8 to 10 individual mice. A significant decrease is seen in the percentage CD4 (upper panels) and CD8 (lower panels) OX40 double-positive T cells in blood. (*P<0.05, Student’s t test).

Antibody Response Against oxLDL
OX40–OX40L interaction plays an important role in T cell dependent humoral responses. B cells differentiate into IgG producing cells after help from OX40-positive, activated T cells.\textsuperscript{14} In this way, OX40-positive T cells induce B cell isotype switching. We determined the isotype specific antibody response to oxLDL in control and anti-OX40L treated mice after 6 weeks of treatment and 8 weeks of Western diet. Figure 4 shows a significant decrease in IgG1 antibodies against oxLDL, whereas IgG2a levels remained constant in treated mice compared with control. This resulted in a very significantly increase in the IgG2a/IgG1 ratio (P<0.001).

Antigen-specific IgG production is preceded by a more general IgM antibody response. Anti-OxLDL specific IgM titers increased significantly in anti-OX40L treated mice compared with control. Even after 2 weeks of treatment (and 4 weeks of Western-type diet) significantly higher IgM levels were observed in treated mice. In addition to an enhanced IgM levels caused by an inhibition of isotype switching after OX40L blockade, the increase in IgM may result from the production of IgM by B1 cells, which is under control of IL-5 (Binder et al\textsuperscript{23}). The concentration of IL-5 in serum of control and treated mice was determined after 6 weeks of treatment and a significant increase in IL-5 levels was determined in anti-OX40L–treated mice (Figure 4).

Th2 Activation Is Inhibited by Anti-OX40L Treatment
Isotype switching of B cells to IgG1 or IgG2a producing cells is dependent on IL secretion by T cells. IL-4 secretion can induce an IgG1 isotype switch, whereas IFN-γ can induce an IgG2a switch. As shown, anti-OX40L antibody treatment resulted in reduced IgG1 levels against OxLDL. We determined the capacity of spleen and peritoneal cells to secrete IFN-γ, IL-4, and IL-5 after CD3/CD28 stimulation for 24 hours (Figure 5). Cells were isolated from mice which received 2 weeks of Western-type diet feeding and anti-OX40L treatment. Two weeks should be sufficient to induce isotype switching, as a normal IgM responses are switched to specific IgG production within 14 days. IL-4 production was significantly decreased after anti-OX40L treatment, whereas IFN-γ production remained unchanged in both spleen and peritoneal leukocytes. IL-5 production by peritoneal cells was reduced, which correlated with the decreased IL-4 production by these cells. In contrast, spleen cells isolated from the same mice secreted increased levels of IL-5 after activation for 24 hours.

Discussion
A recent publication of Wang et al describes the identification of Tnfsf4 (OX40L) as the underlying gene in the Ath-1 QTL\textsuperscript{20,21,24} which renders C57Bl/6J mice susceptible to atherosclerosis in contrast to C3H/Hej and BALB/cJ mice. Mice overexpressing Tnfsf4 showed significantly larger atherosclerotic lesions compared with control, and mice with targeted deletion of this gene had significantly smaller lesions com-
pared with control. Furthermore, the same study showed that humans with a SNP in the OX40L gene have a higher incidence of myocardial infarction.

As Calderhead et al have shown, OX40 expression is very low on naive T cells, and upregulated after activation, we monitored OX40 protein expression on T cells in spleen and blood of LDLr--/ mice fed a Western-type diet. In spleen a significant increase is shown in the number of CD4OX40 and CD8OX40 cells during 2 and 4 weeks of Western-type diet feeding. This increase coincides with an increase of these populations in the circulation. OX40 mRNA levels significantly increased at a later time point than the increase in the percentage of OX40 double-positive cells. The percentage of positive cells, however, does not reflect the expression level of OX40 per cell since it only discriminates between OX40-positive and OX40-negative cells. The difference between mRNA and protein expression of OX40 may reflect the dynamic structure of the spleen and cells expressing high levels of OX40 may be more prone to migration.

The correlation between OX40-positive CD4 and CD8 cells and autoimmune diseases has also been shown for experimental autoimmune encephalomyelitis and GVHD. We show that Western type diet feeding leads to T cell activation based on OX40 expression and OX40 may be an ideal candidate for modulating T cell responses in atherogenesis.

The effect of anti-OX40L antibody treatment on atherosclerosis was investigated in LDLr--/ mice. Treatment led to a significant reduction (53%) in de novo lesion formation. The underlying mechanism of the observed plaque reduction by anti-OX40L treatment may be complex. Not only systemic or local T cell activation, but also B cell function may be involved. Furthermore, very recently Ito et al showed that OX40L strongly inhibited the generation of IL-10 producing type 1 regulatory T cells. These T cells are important in the maintenance of peripheral tolerance and were shown to be protective in the development of atherosclerosis. Future experiments must indicate whether the blockade of OX40L by the anti-OX40L antibodies also results in increased numbers of type 1 regulatory T cells.

Because atherosclerosis is classically considered to be a Th1-mediated disease, we quantified the mRNA levels of IFN-γ and IL-12 in spleens of anti-OX40L treated mice. No significant difference could be observed between the 2 groups, despite the significant reduction in plaque size (supplemental Figure IV) and general T cell activation determined by expression of CD62L and CD69 was not altered between groups (data not shown). We did, however, observe a significant reduction in OX40 mRNA expression in spleen and a decrease in circulating OX40-positive T cells, indicating reduced secondary T cell activation.

OX40 activation has been linked to both Th1 and Th2 activation in several disease models. We determined the capability of spleen and peritoneal cells to produce Th1 (IFN-γ) and Th2 (IL-4, IL-5) cytokines and found a vast and significant decrease in IL-4 secretion after anti-OX40L treatment in these cells, whereas the production of IFN-γ was unaffected. We determined the percentages of T cells in these cell suspensions and no significant differences were observed between the 2 groups.
anti-OxLDL IgM production. These B-1 cells depend on IL-5 for differentiation and maturation. We observed an induction of serum IL-5 in mice treated with anti-OX40L antibodies. IL-5 production by peritoneal cells from anti-OX40L treated mice was significantly decreased, and this is in correlation with the decreased production of IL-4 by these cells after anti-CD3/CD28 activation. In spleen, anti-OX40L treatment leads to increased secretion of IL-5. IL-5 is produced by Th2 cells but because the anti-OX40L–treated mice showed a decrease in Th2 response (lower IL-4 and IgG1), we conclude that other cell types such as mast cells and NK2 cells may be responsible for the increased IL-5 production, and that these cells are (partially) located in the spleen. Because the anti-OX40L treatment resulted not only in a significant increase in IL-5, but also in a trend toward an increased number of CD5 IgM expressing B-1 cells in the peritoneal cavity, we conclude that the increased level of atheroprotective anti-OxLDL IgM on OX40L blockade may also result from B-1 cells in addition to a reduction in isotype switching. Based on the data it is unlikely that the increased oxLDL-specific IgM is derived from the peritoneum, however, migration of activated B-1 cells from peritoneum to germinal centers in the spleen has been reported.

Based on this study we conclude blockade of the OX40/OX40L pathway by an anti-OX40L antibody results in protection against atherosclerosis because of elevated protective IgM titers and a decreased IL-4 production.

Sources of Funding
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Disclosures
None.

References

Because knocking out IL-4 has previously been shown to be protective in atherosclerosis, our data further undermine the hypothesis that Th2 cytokine IL-4 provides protection in atherosclerosis and supports the pro-atherogenic effects of IL-4.

IL-4 is an inducer of IgG1 isotype switching in B cells. In accordance with the reduced IL-4 production by lymphocytes, oxLDL-specific IgG1 levels in mice treated with anti-OX40L were significantly decreased. IFN-γ production was not different in anti-OX40 treated mice compared with control, and correspondingly, IgG2a antibodies against OxLDL were not altered.

An isotype switch toward IgG production is preceded by the production of IgM. The reduced isotype switching to IgG1 as observed in anti-OX40L–treated mice may explain the observed enhanced anti-OxLDL IgM levels. Anti-OxLDL IgM has been shown to provide protection against atherosclerosis by contributing to the elimination of oxidized lipoproteins and cellular debris. In addition to reduced isotype switching, B-1 cells may form another source for

Figure 5. IFN-γ, IL-5 and IL-4 production by spleen and peritoneal cells. Spleen and peritoneal cells isolated from mice that received 2 weeks of Western type diet feeding and treatment with control or anti-OX40L antibody. A significant reduction in IL-4 production is observed in both spleen and peritoneal cells after anti CD3-CD28 stimulation for 24 hours. IL-5 production is significantly decreased by peritoneal cells treated with anti-OX40L antibody, but is significantly decreased in spleen cell cultures from these mice. IFN-γ production is not affected by anti-OX40L treatment. White bars represent control mice (n=5), black bars represent anti-OX40L treated mice (n=5). Error bars represent SEM. *P<0.05, **P<0.01.


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Online figure I. mRNA expression of T cell activation markers in the spleen in response to a Western type diet

mRNA was isolated from spleen using the GTC method and expression of the indicated genes is expressed relative to 36B4 and HPRT, and subsequently related to the expression in mice on chow diet. An unpaired Student t test was applied to test whether mRNA levels were significantly different from the mRNA levels in chow fed animals (*p < 0.05, n= 6 per time point).

Online figure II. Anti OX40L antibody treatment reduces collar induced atherosclerosis

Mice received treatment with 300 µg of anti OX40L (α-OX40L) or control rat IgG in 100 µl of sterile PBS twice a week during 6 weeks. A marked and significant decrease was seen for intima/lumen ratio (A), and intima / media ratio (B). Nuclei density of the plaque significantly decreased after treatment (C). Error bars represent SEM, n = 10 per group (*=P<0.05, Mann Whitney test).

Online figure III. Anti OX40L antibody treatment has no effect on collagen and macrophage positive area

Plaque collagen content is stained with Pico Sirius Red in control (A) and treated (B) mice. Relative collagen area does not differ between groups (C). Relative macrophage staining of control (D) and treated mice (E) is visualized using a monoclonal antibody specific for macrophages (MOMA-2). No difference in the percentage MOMA positive area is observed between control and treated mice (F).
Online figure IV. Determination of OX40 levels on CD4 and CD8 positive T cells
Mononuclear cell suspensions from spleen and blood were isolated from control mice and mice following 0, 2 or 4 weeks of western type diet feeding. Representative plots for a CD8/OX40 double stain in week 0 and 4 are shown. Isotype control resulted in negative staining.

Online figure V. Spleen mRNA expression of Th1 inflammatory markers after anti OX40L treatment
mRNA expression of different genes in the spleen of anti OX40L treated mice (6 weeks) is expressed relative to 36B4 and HPRT, and subsequently related to the expression in control IgG treated mice. White bars represent control mice (n=10), black bars represent anti-OX40L treated mice (n=10). An unpaired Student t test was applied to test if mRNA levels were significantly different from the mRNA levels in chow fed animals (*P<0.05).

Figure VI. CD5+IgM+ cells are increased in the peritoneum of mice treated with anti-OX40L
Peritoneal and spleen cells were isolated from mice which received 300 µg of anti-OX40L or control rat IgG in 100 µl of sterile PBS twice a week during 2 weeks. CD5 and IgM expression were determined using FACS cytometry and double positive cells within lymphocyte population were determined. (n=5-6 mice per group).
### Table I: PCR primers

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### Table II: Serum lipid levels at sacrifice

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<th>anti-OX40L treated mice</th>
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<td>Total Cholesterol (mg/ml)</td>
<td>1037 ± 45</td>
<td>905 ± 55</td>
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<tr>
<td>Triglycerides (mg/ml)</td>
<td>646 ± 30</td>
<td>538 ± 20</td>
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<tr>
<td>Weight (g)</td>
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<td>26.8 ± 0.7</td>
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Online figure II

Graphs showing the Intima / Lumen ratio, Intima / Media ratio, and Nuclei/µm plaque surface for Control and α-OX40L groups. The graphs indicate statistically significant differences marked with asterisks (*) between the groups.
Online figure III

Collagen / plaque ratio
Macrophage / plaque ratio

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<th>Macrophage / plaque ratio</th>
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<td>α-OX40L</td>
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Online figure IV

Gate for CD8⁺ or CD4⁺ cells

Isotype control

Week 0

Week 4

CD8-FITC

FCS

OX40-PE

CD8-FITC

CD8-FITC

13%

61%

0.3%
Online figure V

![Graph showing relative expression of IL-12 and IFN-γ with control and α-OX40L conditions.](image-url)