# Vascular Medicine

# Induction of Oral Tolerance to Oxidized Low-Density Lipoprotein Ameliorates Atherosclerosis

G.H.M. van Puijvelde, MSc; A.D. Hauer, PhD; P. de Vos, BSc; R. van den Heuvel, BSc;
M.J.C. van Herwijnen, BSc; R. van der Zee, PhD; W. van Eden, PhD; T.J.C. van Berkel, PhD; J. Kuiper, PhD

***Background***—Oxidation of low-density lipoprotein (LDL) and the subsequent processing of oxidized LDL (oxLDL) by macrophages results in activation of specific T cells, which contributes to the development of atherosclerosis. Oral tolerance induction and the subsequent activation of regulatory T cells may be an adequate therapy for the treatment of atherosclerosis.

***Methods and Results***—Tolerance to oxLDL and malondialdehyde-treated LDL (MDA-LDL) was induced in LDL receptor$^{-/-}$ mice fed a Western-type diet by oral administration of oxLDL or MDA-LDL before the induction of atherogenesis. Oral tolerance to oxLDL resulted in a significant attenuation of the initiation (30% to 71%; $P<0.05$) and progression (45%; $P<0.05$) of atherogenesis. Tolerance to oxLDL induced a significant increase in CD4$^+$CD25$^+$Foxp3$^+$ cells in spleen and mesenteric lymph nodes, and these cells specifically responded to oxLDL with increased transforming growth factor-$\beta$ production. Tolerance to oxLDL also increased the mRNA expression of Foxp3, CTLA-4, and CD25 in the plaque. In contrast, tolerance to MDA-LDL did not affect atherogenesis.

***Conclusions***—OxLDL-specific T cells, present in LDL receptor$^{-/-}$ mice and important contributors in the immune response leading to atherosclerotic plaque, can be counteracted by oxLDL-specific CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells activated via oral tolerance induction to oxLDL. We conclude that the induction of oral tolerance to oxLDL may be a promising strategy to modulate the immune response during atherogenesis and a new way to treat atherosclerosis. (***Circulation***. 2006;114:1968-1976.)

***Key Words:*** atherosclerosis ■ immune system ■ T cells ■ tolerance induction

The uptake of oxidized low-density lipoprotein (oxLDL) in the vessel wall by antigen presenting cells (APCs), such as macrophages and dendritic cells, is one of the hallmarks of the T helper 1 (Th1)–mediated immune response in atherosclerosis. OxLDL ingested by macrophages is processed, and oxLDL-derived epitopes will be presented on the cell surface via major histocompatibility complex (MHC) class I and II molecules. Via the T cell receptor, oxLDL-specific CD8$^+$ and CD4$^+$ T cells bind to MHC class I or II antigen complex, respectively, and this results in the activation and proliferation of oxLDL-specific T cells. The putative role of this oxLDL-specific response in atherosclerosis is established by the presence of oxLDL-specific T cells in the vessel wall and atherosclerotic plaques.[1,2] These T cells are in particular Th1 cells producing Th1 cytokines such as interferon-$\gamma$ (IFN-$\gamma$), interleukin (IL)-12, IL-2, and tumor necrosis factor-$\alpha$ (TNF-$\alpha$).[3–5] In addition, immunoglobulin G2a (IgG2a) antibodies against oxLDL, indicating Th1 assistance, predominate in plasma of low-density lipoprotein

## Editorial p 1901
## Clinical Perspective p 1976

receptor (LDLr)$^{-/-}$ and apolipoprotein E (apoE)$^{-/-}$ mice during early stages of atherosclerosis.[6] The presence of antibodies to oxLDL in serum of patients with cardiovascular diseases also proves that oxLDL is one of the (auto)antigens in atherosclerosis.[7]

Oral tolerance induction to autoantigens is one of the possible treatments for Th1-mediated autoimmune diseases such as multiple sclerosis,[8,9] rheumatoid arthritis,[10,11] and type 1 diabetes.[12,13] Antigens administered orally enter the gut-associated lymphoid tissue (GALT),[14,15] which, as a primary function, protects the host from ingested pathogens and proteins.[16,17] Currently, 2 primary effector mechanisms of oral tolerance induction are known. Oral administration of high doses of an antigen result in anergy or deletion of antigen-specific T cells,[18,19] whereas low doses of an antigen result in the induction of antigen-specific regulatory T cells.[20] These regulatory T cells can be divided into 3 groups: Th3

cells and Tr1 cells, mediating suppression via secretion of transforming growth factor-$\beta$ (TGF-$\beta$) and IL-10, respectively, and CD4$^+$CD25$^+$ regulatory T cells characterized by the expression of the transcription factor forkhead box P3 (Foxp3). The regulatory function of the CD4$^+$CD25$^+$Foxp3$^+$ cell is mediated by cell contact and surface-bound TGF-$\beta$ and cytotoxic T lymphocyte–associated antigen-4 (CTLA-4).[21] After activation in the GALT, the regulatory T cells migrate to the site of inflammation, and on reencountering the fed antigen they display their specific suppressive effect, resulting in an attenuated Th1-mediated immune response specific for the fed antigen.

Initial studies show that oral tolerance induction to $\beta$2-glycoprotein I[22] and HSP65[23,24] results in the suppression of early atherosclerosis and demonstrate that oral tolerance induction could be a successful treatment for atherosclerosis.

In the present study we demonstrate that induction of oral tolerance to oxLDL attenuates both the initiation and the progression of atherosclerosis, whereas malondialdehyde-treated LDL (MDA-LDL) was unable to mediate this effect. The effect of oxLDL tolerance may be explained by a significant increase in CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in the mesenteric lymph nodes and spleen, an increased production of TGF-$\beta$ by these cells, and a significant upregulated expression of Foxp3 and CD25 in the atherosclerotic lesions.

## Methods

### Animals

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice were from the Jackson Laboratory, Bar Harbor, Me. They were kept under standard laboratory conditions and administered food and water ad libitum.

### Antigens and Adjuvant

Dimethyl dioctadecyl ammonium bromide (DDA) was from Sigma Diagnostics, St Louis, Mo. LDL was isolated from serum of a healthy volunteer.[25] Isolated LDL was oxidized by 10 $\mu$mol/L CuSO$_4$ at 37°C for 20 hours,[26] and MDA-LDL was made by addition of 0.5 mol/L MDA to 10 mg of LDL for 3 hours at 37°C.

### Immunizations

LDLr$^{-/-}$ mice were immunized with 100 $\mu$g of oxLDL or MDA-LDL together with 100 $\mu$g of DDA via 1 intraperitoneal injection. After 14 days, spleens were used in proliferation assays.

For details of the spleen cell proliferation assay, plaque analysis, polymerase chain reaction assays, and flow cytometric analysis, see the online-only Data Supplement.

### Induction of Atherosclerosis

Atherosclerosis was induced in LDLr$^{-/-}$ mice by feeding them a Western-type diet (0.25% cholesterol and 15% cocoa butter; Special Diet Services, Witham, Essex, UK) 2 weeks before placement of perivascular collars.[27] Total cholesterol levels were quantified by an enzymatic procedure (Roche Diagnostics, Mannheim, Germany) with Precipath used as an internal standard.

### Oral Tolerance Induction During the Induction of Atherosclerosis

To induce tolerance, LDLr$^{-/-}$ mice were fasted for 16 hours. Then 2 mg of soybean trypsin inhibitor (Sigma) was administered orally to prevent antigen degradation; 10 minutes thereafter, mice orally received phosphate-buffered saline (PBS), 30 $\mu$g of superoxide dismutase (Sigma), or 30 $\mu$g of oxLDL or MDA-LDL. Injections were repeated 3 times to a total of 4 injections in 8 days. After the antigens were administered, mice were kept on a Western-type diet. These procedures were performed either in the first week of diet or after 10 weeks of diet.

### Detection of Anti-oxLDL Antibodies

OxLDL (5 $\mu$g/mL) dissolved in a NaHCO$_3$/Na$_2$CO$_3$ buffer (pH 9.0) was coated. Measurement of IgG1 and IgG2a levels in serum was performed with an ELISA Ig detection kit (Zymed Laboratories, San Francisco, Calif) in a manner that conformed to the manufacturer's protocol, and appropriate controls were performed.

### Cytokine Assays

Mesenteric lymph node cells were cultured at $2\times10^6$ cells per milliliter with or without 5 $\mu$g/mL oxLDL. IL-10, IFN-$\gamma$ (eBioscience, San Diego, Calif), and TGF-$\beta$ (Bender MedSystems, Vienna, Austria) concentrations in the supernatants were determined by ELISA.

### Statistical Analysis

All data are expressed as mean±SEM. The 2-tailed Student $t$ test was used to compare proliferative responses to antigens, fluorescence-activated cell sorter data, differences in cytokine production, and atherosclerotic parameters between the different groups.

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

## Results

### T Cells Specific for OxLDL and MDA-LDL in LDLr$^{-/-}$ Mice

The presence of T cells specific for oxLDL and MDA-LDL epitopes in LDLr$^{-/-}$ mice was investigated via a spleen cell proliferation assay. Splenocytes isolated from naive LDLr$^{-/-}$ mice were incubated with several concentrations of oxLDL or MDA-LDL. Low concentrations of oxLDL, 1 and 5 $\mu$g/mL, resulted in a 1.53±0.17-fold ($P<0.05$) and 2.52±0.23-fold ($P<0.001$) increase in proliferation, respectively (Figure 1A). In case of MDA-LDL, incubation of splenocytes with 1, 5, and 10 $\mu$g/mL of MDA-LDL resulted in a 1.62±0.15-fold ($P=$NS), 2.90±1.15-fold ($P<0.05$), and 4.41±0.89-fold ($P<0.01$) increase in proliferation, respectively (Figure 1B). OxLDL was toxic at concentrations $>10$ $\mu$g/mL, whereas MDA-LDL was only toxic at concentrations $>100$ $\mu$g/mL (data not shown). In all experiments, concanavalin A, a general pan T cell activator, induced a $>50$-fold increase in proliferation (data not shown).

In addition, we determined whether the T cell response to modified LDL can be modulated in vivo. LDLr$^{-/-}$ mice were immunized by intraperitoneal injection of 100 $\mu$g of oxLDL or MDA-LDL in combination with the adjuvant DDA. Two weeks thereafter, the mice were euthanized and isolated splenocytes were incubated with oxLDL or MDA-LDL. OxLDL induced a higher proliferation at 1 and 5 $\mu$g/mL of 2.89±0.29-fold and 7.25±0.81-fold, respectively, compared with the controls ($P<0.01$) (Figure 1C). Incubation of splenocytes isolated from MDA-LDL–immunized mice with 1, 5, and 10 $\mu$g/mL MDA-LDL resulted in a 2.07±0.82-fold ($P=$NS), 3.94±0.41-fold ($P<0.01$), and 6.17±1.50-fold ($P<0.05$) increase in proliferation (Figure 1D).
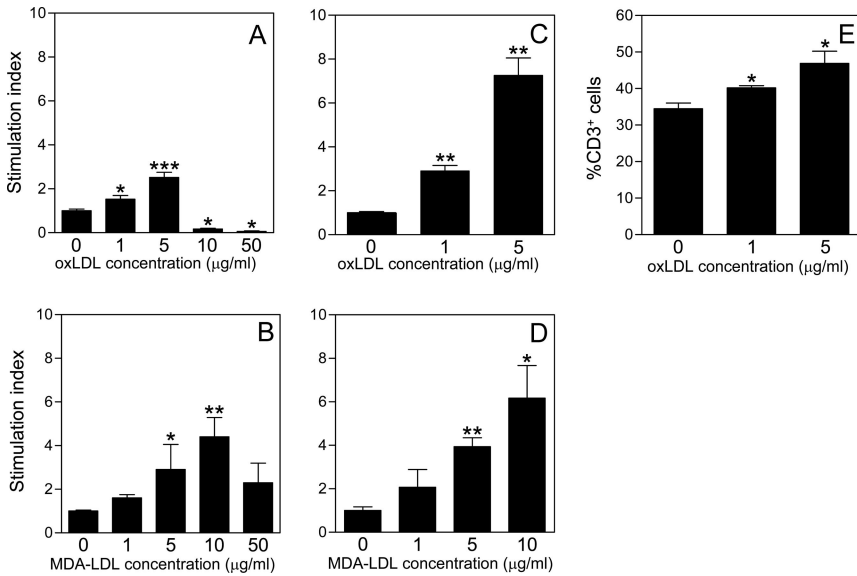
**Figure 1.** Spleen cell proliferation in response to oxLDL and MDA-LDL. LDLr$^{-/-}$ mice were immunized via 1 intraperitoneal injection with 100 $\mu$g of oxLDL or MDA-LDL. Two weeks thereafter, splenocytes were isolated from naive and immunized LDLr$^{-/-}$ mice and cultured with oxLDL (A and C, respectively) or MDA-LDL (B and D, respectively) for 24 hours. In the control situation, splenocytes from naive or immunized mice were cultured in the absence of oxLDL or MDA-LDL. The amount of proliferation was measured by incorporation of [$^3$H]thymidine, which was added for the final 16 hours of the assay. Data are shown as the stimulation index $\pm$SEM. The stimulation index is defined as the ratio of the mean counts per minute of triplicate cultures with antigen to the mean counts per minute in culture medium without antigen. E, Percentage of CD3$^+$ cells (mean$\pm$SEM) after proliferation of the splenocytes with 1 and 5 $\mu$g/mL of oxLDL. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

A flow cytometric analysis was performed on the proliferating cells to determine the cell type responsible for the cell proliferation. The amount of CD3$^+$ T cells increased significantly from 34.5$\pm$1.5% to 46.9$\pm$2.4% when splenocytes were incubated with 5 $\mu$g/mL of oxLDL (Figure 1E; $P<0.05$). The amount of macrophages was not affected by the incubation with oxLDL (data not shown).

### Effect of Oral Tolerance Induction to OxLDL and MDA-LDL on Initiation of Atherosclerosis

To determine the effect of oral tolerance induction to oxLDL and MDA-LDL on atherosclerotic plaque initiation, atherosclerosis was induced after oral administration of oxLDL and MDA-LDL to LDLr$^{-/-}$ mice. Mice were put on a Western-type diet for 1 week, and subsequently oral tolerance was induced by oral administration of 30 $\mu$g of oxLDL or MDA-LDL. Treatment was repeated every other day to 4 times in total. After tolerance induction, the Western-type

diet feeding was continued, and 1-week later mice were equipped with collars around both carotid arteries to induce atherosclerosis.[28] During the experiment, total plasma cholesterol levels were not significantly different between the groups and increased from 679$\pm$64 (before diet) to 1554$\pm$140 mg/dL in control mice to 1755$\pm$146 mg/dL in oxLDL-treated mice and to 1718$\pm$77 mg/dL in MDA-LDL–treated mice. Six weeks after collar placement, atherosclerotic plaque formation was analyzed after a hematoxylin-eosin staining of cryosections of the carotid arteries (Figure 2A, 2B). Oral feeding of LDLr$^{-/-}$ mice with superoxide dismutase had no effect on plaque size compared with PBS (data not shown). Oral tolerance induction to oxLDL resulted in a significant 71.2% reduction in plaque area (Figure 2C; 20 768$\pm$5964 versus 6046$\pm$1941 $\mu$m$^2$; $P<0.05$) compared with control mice. Furthermore, a beneficial 55.3% reduction in intima/lumen ratio (Figure 2D; 0.25$\pm$0.06 versus 0.11$\pm$0.03; $P<0.05$) and a 72.1% reduction in intima/media
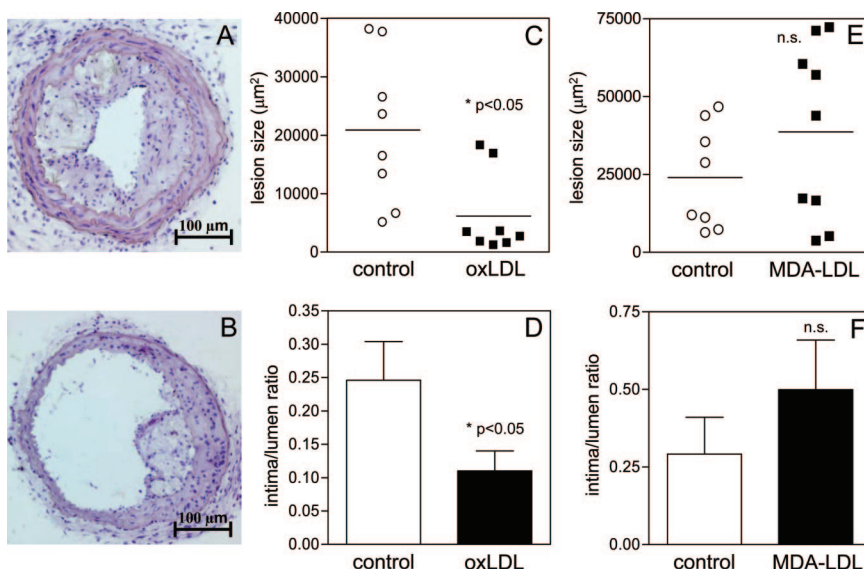


**Figure 2.** Effect of oral tolerance induction to oxLDL on atherosclerotic plaque formation in collar-induced atherosclerosis in the carotid artery. LDLr$^{-/-}$ mice were fed oxLDL or MDA-LDL 4 times before atherosclerosis was induced by collar placement around both carotid arteries. Six weeks after collar placement, the mice were killed, and the carotid arteries of control-treated (A), oxLDL-treated (B), and MDA-LDL–treated (not shown) mice were sectioned and stained with hematoxylin-eosin. The lesions were quantified by computer-assisted morphometric analysis, and the plaque size and intima/lumen ratio of oxLDL-treated (C and D, respectively) and MDA-LDL–treated mice (E and F, respectively) were determined.
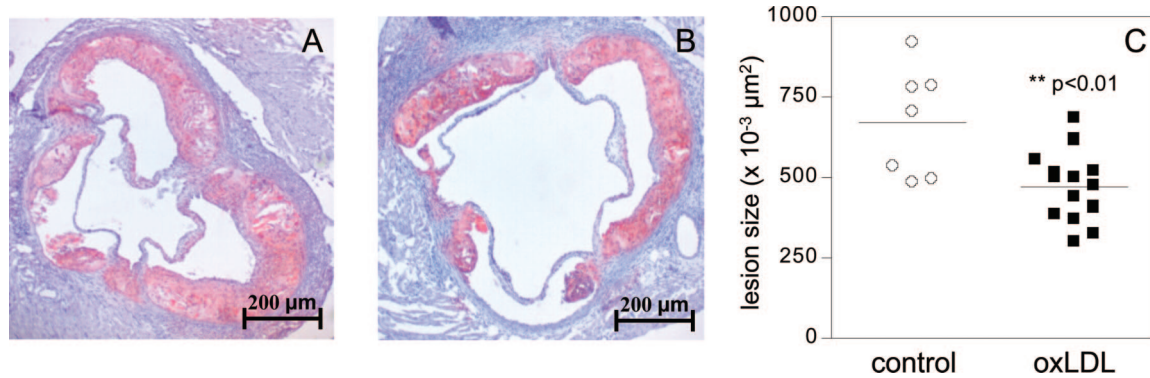
**Figure 3.** Effect of oral tolerance induction to oxLDL on atherosclerotic plaque formation in the aortic root. LDLr$^{-/-}$ mice were fed oxLDL 4 times before atherosclerosis was induced by feeding a Western-type diet. After 8 weeks of diet, mice were killed, and the aortic roots of control-treated (A) and oxLDL-treated (B) mice were sectioned and stained with Oil red O and hematoxylin. The lesions were quantified, and the plaque size was determined (C).

ratio (data not shown; $0.75\pm0.23$ versus $0.21\pm0.04$; $P<0.05$) were observed. In the case of MDA-LDL, oral tolerance induction did not result in a significant effect on plaque size (Figure 2E; $23\,314\pm8016$ versus $37\,327\pm13\,137$ $\mu$m$^2$; $P=0.41$), and no significant effect on intima/lumen ratio (Figure 2F; $0.29\pm0.12$ versus $0.50\pm0.16$; $P=0.34$) and intima/media ratio (data not shown; $0.42\pm0.15$ versus $1.12\pm0.34$ in the MDA-LDL–treated group; $P=0.12$;) was observed. In a second independent experiment, the effect on plaque formation in the aortic root was determined at 8 weeks of diet. Oral treatment with oxLDL (Figure 3B) resulted in a significant 29.9% reduction in plaque size (Figure 3C; $669\,243\pm56\,643$ versus $469\,288\pm27\,950$ $\mu$m$^2$; $P<0.01$) compared with control-treated mice (Figure 3A). Immunohistochemical analysis of the plaques of both experiments showed that oral tolerance induction to oxLDL and MDA-LDL had no effect on the relative macrophage and smooth muscle cell content compared with plaques of control mice (data not shown).

## Effect of Oral Tolerance Induction to OxLDL on Progression of Atherosclerosis

Next we determined the effect of tolerance induction to oxLDL on the progression of atherosclerosis. To this end, LDLr$^{-/-}$ mice were put on a Western-type diet for 10 weeks, which resulted in the initial formation of plaques in the aortic root. At that time point, oral tolerance to oxLDL was induced (30 $\mu$g of oxLDL, 4 times), and subsequently the LDLr$^{-/-}$ mice were kept on a Western-type diet for another 7 weeks. During the experiment, total plasma cholesterol levels increased from $503\pm67$ to $2727\pm243$ mg/dL and $2422\pm366$ mg/dL in the control and oxLDL-treated mice, respectively (data not shown; $P=$NS). After 7 weeks, the mice were euthanized, cryosections of the aortic root of control-treated (Figure 4A) and oxLDL-treated (Figure 4B) mice were stained with Oil red O, and atherosclerotic plaque formation in the aortic root was analyzed. OxLDL-treated mice showed a modest but significant 24.3% reduction in plaque size at the aortic root (Figure 4C; $715\,523\pm56\,365$ versus $541\,353\pm55\,239$ $\mu$m$^2$; $P<0.05$) compared with control-

treated mice. The effect of oral tolerance induction to oxLDL is more impressive when one takes into account that the size of the plaques at the time of tolerance induction was 300 000 $\mu$m$^2$ in a third group of mice. Subtraction of the lesion size at the start of the oral feeding establishes that oxLDL treatment led to a 42.4% reduction in plaque progression (Figure 4D). Interestingly, no effect of tolerance induction to oxLDL was observed on the relative macrophage and smooth muscle cell content in the plaque (data not shown).

## Effect of Oral Tolerance Induction to OxLDL on CD4$^+$CD25$^+$Foxp3$^+$ Regulatory T Cells

To determine whether oral tolerance induction to oxLDL was associated with a change in regulatory T cell levels, a flow cytometric analysis was performed. CD4$^+$CD25$^+$Foxp3$^+$ cells are normally present in low numbers in spleen ($0.8\pm0.2$%), mesenteric lymph nodes ($3.0\pm0.4$%), Peyer's patches ($1.7\pm0.4$%), and blood ($1.7\pm0.1$%) of control LDLr$^{-/-}$ mice. Two and 4 days after the fourth and last oral feeding of oxLDL, the number of CD4$^+$CD25$^+$Foxp3$^+$ cells increased significantly to $1.3\pm0.1$% and $1.6\pm0.2$% in the spleen, respectively (Figure 5A; $P<0.05$) and to $5.2\pm0.2$% and $5.8\pm0.6$% in mesenteric lymph nodes, respectively (Figure 5B; $P<0.01$). No significant changes were seen in the Peyer's patches and blood. To determine whether the effect was long lasting, mice were euthanized 14 days after the last oral feeding. CD4$^+$CD25$^+$Foxp3$^+$ cells were still increased in spleen and mesenteric lymph nodes ($1.4\pm0.1$% [$P<0.05$] and $5.4\pm0.3$% [$P<0.01$], respectively) after oral feeding of oxLDL (Figure 5A, 5B), whereas MDA-LDL did not affect the number of CD4$^+$CD25$^+$Foxp3$^+$ cells (not shown). To determine the effect of induction of CD4$^+$CD25$^+$Foxp3$^+$ cells on cytokine induction, mesenteric lymph nodes of control-, oxLDL-treated, and MDA-LDL–treated mice were restimulated with 5 $\mu$g/mL oxLDL or MDA-LDL in vitro. We observed that oxLDL only induced a significant (6-fold) increase in TGF-$\beta$ in lymph node cells from oxLDL-tolerant mice but not in control-pretreated mice, whereas MDA-LDL was unable to induce TGF-$\beta$ in MDA-LDL–pretreated or
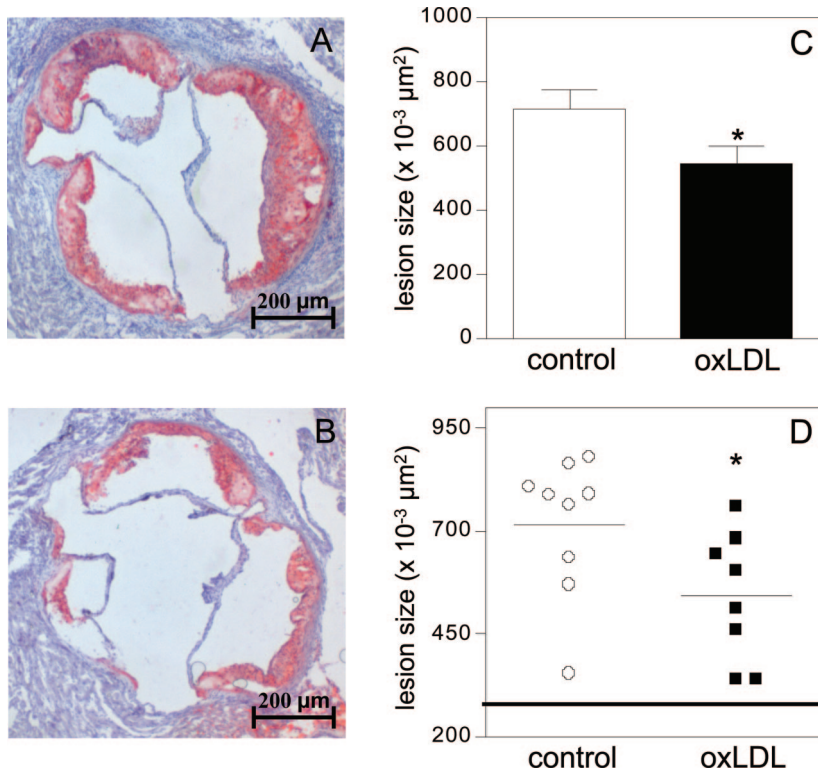
**Figure 4.** Oral tolerance induction to oxLDL attenuates atherosclerotic plaque progression in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks before oxLDL was administered orally 4 times. After oral tolerance induction, the mice were kept on a Western-type diet for 7 more weeks. The aortic roots of control-treated (A) and oxLDL-treated (B) mice were sectioned and stained with Oil red O and hematoxylin. Lesions at the aortic root were quantified by computer-assisted morphometric analysis, and the plaque size (C) was determined. D, Dot plot of lesion size in all mice. The bar at $0.30 \times 10^6$ $\mu m^2$ represents the lesion size of mice after 10 weeks of diet without oral treatment. *$P < 0.05$.

control mice (not shown). The IL-10 and IFN-γ levels were below the detection limits in any of the experiments.

## Expression of Regulatory T Cell Markers in Atherosclerotic Plaques

We analyzed the expression of CD25 and Foxp3 in atherosclerotic plaques in the carotid arteries. After treatment with oxLDL (n=14) and 8 weeks of Western-type diet feeding, the relative mRNA expression of Foxp3, CTLA-4, and CD25 was significantly upregulated in the atherosclerotic plaque compared with control mice (n=8). Foxp3 showed a 1.5-fold increase, CTLA-4 a 1.7-fold increase, and CD25 a 2.2-fold increase (Figure 6; $P < 0.05$).

## Influence of Oral Tolerance Induction to OxLDL on IgG Patterns

OxLDL-specific IgG1 and IgG2a levels in serum were determined at the end of the experiment on initiation of atherosclerosis. No detectable differences in IgG1 and IgG2a levels were observed (Figure 7A and 7B, respectively), and no difference between the IgG1/IgG2a ratio in control and oxLDL-treated mice was observed (Figure 7C).

## Discussion

One of the first events in atherosclerosis is the oxidative modification of LDL and the subsequent uptake of oxLDL by macrophages. Epitopes from oxLDL, such as apoB-100 peptides[29] and oxidized phosphorylcholine-containing phospholipids,[30] can be presented by APCs and cause T cell activation.[31] Autoreactive T cells specific for oxLDL epitopes have been found in human atherosclerotic plaques[1] and in atherosclerotic lesions in apoE$^{-/-}$ mice.[2]

We now demonstrate that naive LDLr$^{-/-}$ mice already contain T cells specific for oxLDL and MDA-LDL, as shown in spleen cell proliferation assays. In addition, we show that the in vivo response to oxLDL and MDA-LDL can be modified by immunization with oxLDL or MDA-LDL. The spleen cell proliferation showed an enhanced proliferation (3- to 7-fold) in the immunized mice compared with the naive mice.

It is well known that the T cells in atherosclerotic lesions, reactive to several antigens such as oxLDL and heat shock proteins, mainly produce Th1 cytokines, resulting in a disturbed balance between Th1 and Th2 cytokines. Several studies show that the extent of atherosclerosis can be reduced via Th1 cytokine inhibition.[32–35] In addition, stimulation of the Th2 cytokine production can attenuate atherosclerosis.[28,36] Although restoration of the imbalance between Th1 cells and Th2 cells may be effective in treating atherosclerosis, some debate on the beneficial role of Th2 cells in atherosclerosis exists: Experiments with IL-4, a Th2 cytokine, show that IL-4 may be proatherogenic.[32] Mallat et al[37] hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and/or Th2) and regulatory T cells specific for "altered" self and nonself antigens. Recently, Ait-Oufella et al[38] showed that regulatory T cells play an important role in controlling the development of atherosclerosis. A possible mechanism to achieve a beneficial shift in the balance between pathogenic T cells and regulatory T cells is mucosal tolerance induction. The regulatory T cells induced via nasal tolerance, Tr1 cells, mainly produce IL-10, and regulatory T cells induced via oral tolerance, Th3 cells, mainly produce TGF-β.[39] Besides Tr1 and Th3 cells, mucosal tolerance induction can also lead to activation of
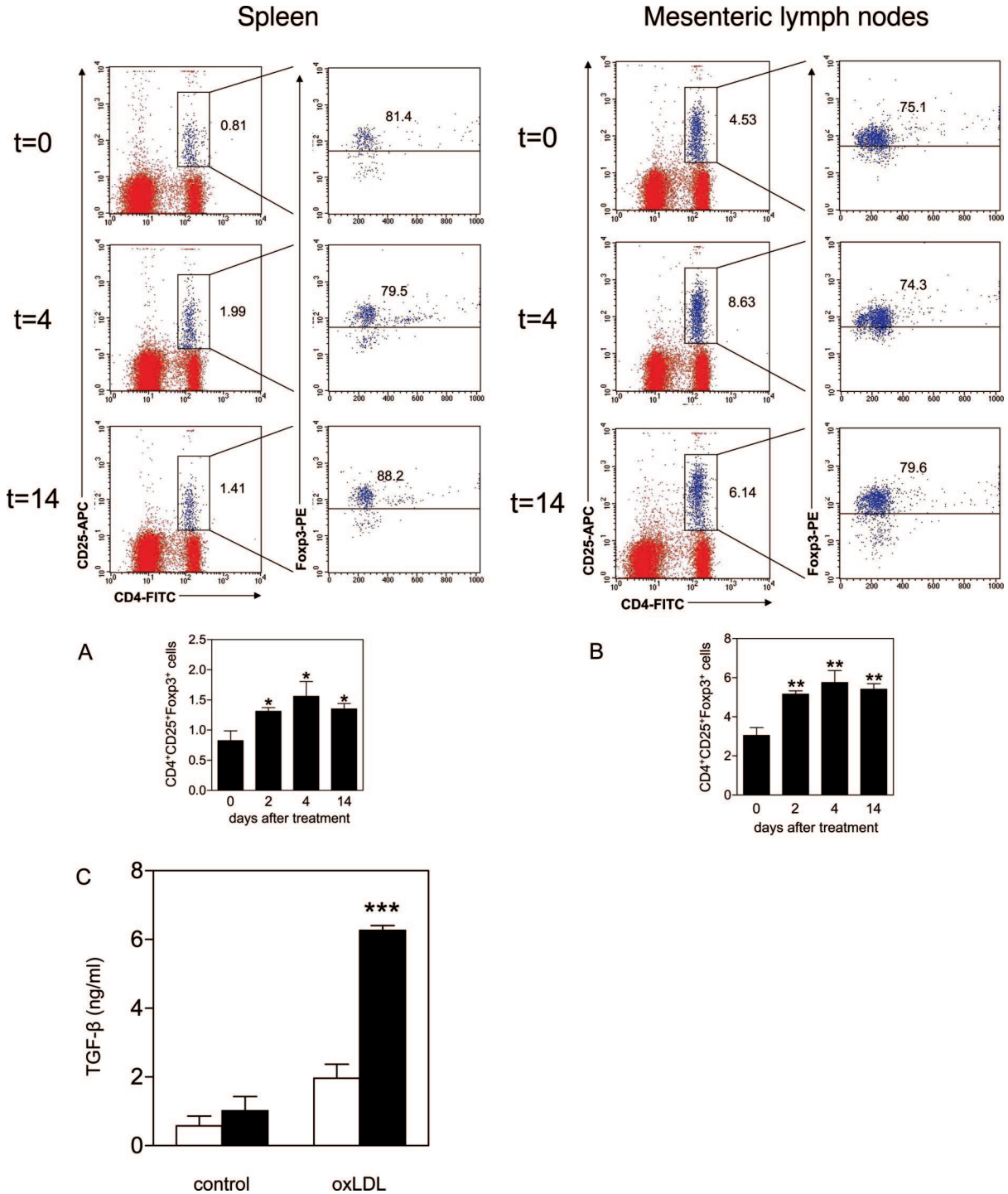
**Figure 5.** Effect of oral tolerance induction to oxLDL on CD4$^+$CD25$^+$Foxp3$^+$ cells in spleen and mesenteric lymph nodes. LDLr$^{-/-}$ mice were fed PBS or oxLDL 4 times and were killed at days 2, 4, and 14. The dot plots show representative examples of lymphoid cells isolated from the spleen (A) and mesenteric lymph nodes (B) stained for CD4 and CD25 (left). The right panel of dot plots shows the percentage of Foxp3$^+$ cells within the CD4$^+$CD25$^+$ population. The graphs represent the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells (mean±SEM). *$P<0.05$, **$P<0.01$. C, TGF-$\beta$ production by mesenteric lymph node cells isolated 14 days after treatment from control- and oxLDL-treated mice and restimulated with PBS (open bars) or oxLDL (closed bars) in vitro. ***$P<0.001$ (compared with all other bars).

CD4$^+$CD25$^+$Foxp3$^+$ cells. Foxp3 is known as an exclusive marker for natural regulatory T cells.[40] The regulatory function of these cells is mediated by cell contact and surface-bound TGF-$\beta$ and CTLA-4.[21]

In our present study, we show that oral tolerance induction to oxLDL can attenuate atherosclerosis in both an early stage and an advanced stage. A relatively low dose of oxLDL (30 $\mu$g, 4 times) significantly attenuated early atherosclerotic lesion formation in the carotid arteries by 71.2% and at the aortic root by 29.9%. The effect on lesion initiation is reflected in the intima/lumen ratio (55.3% reduction) and the intima/media ratio (72.1% reduction) of carotid arteries. Our
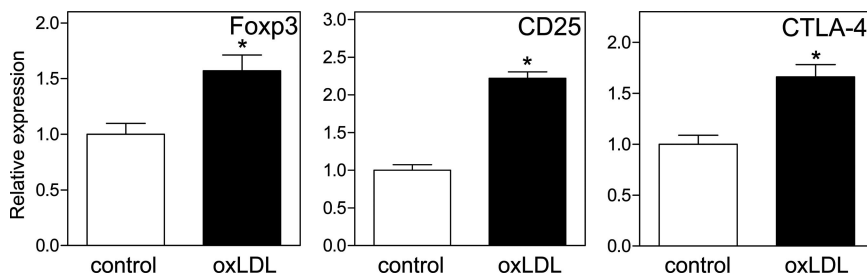
**Figure 6.** Expression of regulatory T cell markers in control- and oxLDL-treated mice. mRNA was isolated from carotid arteries of control- (n=8) and oxLDL-treated (n=14) mice, and the mRNA levels of Foxp3, CTLA-4, and CD25 were quantitatively determined and expressed relative to 36B4. *P<0.05.

results are in agreement with studies that showed that oral or nasal tolerance induction to HSP65 and $\beta$2-glycoprotein I may be a useful treatment for atherosclerosis.[22–24] In our present study, a significant 24.3% reduction in advanced atherosclerotic lesion size was also observed. When we take the initial lesion size into account, a 42.4% reduction in plaque progression is obtained. In agreement with the studies on HSP65 and $\beta$2-glycoprotein I, no effect on the relative macrophage and collagen content of the plaque was seen in both experiments.[22,24] We have previously shown that over-expression of IL-10, another way to modulate the inflammatory process in atherosclerosis, largely reduced (62.2%) the atherosclerotic lesion formation without any effect on macrophage, collagen, and smooth muscle cell content of the plaque.[28]

In addition to oxLDL, MDA-LDL was used in this study as antigen for oral tolerance induction. Surprisingly, no significant effect on atherosclerotic plaque area was seen after oral tolerance induction with 30 $\mu$g of MDA-LDL in LDLr$^{-/-}$ mice. The LDLr$^{-/-}$ mice treated orally with MDA-LDL showed a 60.8% (P=0.41) increase in plaque area and a 71.1% (P=0.34) increase in intima/lumen ratio.

We also explored the possible effect of oral tolerance induction on the antibody isotype distribution. No significant alterations in oxLDL-specific IgG1 and IgG2a levels were detected, and consequently no effect on the IgG1/IgG2a ratio was observed, suggesting that the Th1/Th2 ratio in the mice was not altered by tolerance induction.

The lack of an effect on oxLDL-specific antibodies demonstrates that the suppressing effects shown after oral tolerance induction are not caused by an effect on the humoral immune response. In the majority of the studies and in all studies on atherosclerosis, the exact mechanism behind oral tolerance is still unclear. Increased levels of IL-10 and TGF-$\beta$ after oral tolerance induction are caused by the stimulated development of adaptive immune cells.[24] Zhang et al[41] found an increased amount of CD4$^+$CD25$^+$ cells after feeding mice with ovalbumin. These regulatory T cells were declared to be responsible for the high levels of IL-10 and TGF-$\beta$. Recently, CD4$^+$CD25$^+$ cells with Foxp3 expression were found to be immunosuppressive. We demonstrate that oxLDL tolerance induction significantly increases the number of CD4$^+$CD25$^+$Foxp3$^+$ cells for up to 2 weeks in the spleen and the mesenteric lymph nodes. Because these regulatory T cells exert their suppressive effect via cell-cell contact and surface-bound TGF-$\beta$, we determined the TGF-$\beta$ production in the mesenteric lymph nodes. Lymph node cells from oxLDL-tolerant mice produced 6-fold higher levels of TGF-$\beta$ on restimulation with oxLDL than cells from control mice, indicating the induction of TGF-$\beta$–producing regulatory T cells. In addition, analysis of mRNA expression levels showed that oxLDL tolerance induction increased the expression of Foxp3, CTLA-4, and CD25 within the plaque, clearly indicating the presence of regulatory T cells within the lesions on tolerance induction. The profile of gene expression on oxLDL tolerance induction, together with the induction of Foxp3 in spleen and lymph nodes and concurrent oxLDL-specific TGF-$\beta$ production in the absence of IL-10 and IFN-$\gamma$ production, strongly suggests that CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells rather than Tr1 or Th3 are responsible for the observed effects of oxLDL tolerance induction on atherosclerosis. The absence of induction of regulatory T cells by MDA-LDL and the absence of TGF-$\beta$ induction in lymph node cells of MDA-LDL–pretreated cells by MDA-LDL may explain the absence of a regulatory effect of oral treatment with MDA-LDL.

It may be speculated that oxLDL-specific regulatory T cells induced by oral tolerance regulate the action of oxLDL-specific CD4$^+$ T cells within the plaque in the following way: OxLDL-specific CD4$^+$ T cells are activated within the plaque by oxLDL-presenting APCs, which may lead to the expression of TGF-$\beta$ receptor II (T$\beta$ RII) by these cells. OxLDL-specific regulatory T cells also recognize oxLDL presented by the APCs and via the enhanced production of TGF-$\beta$
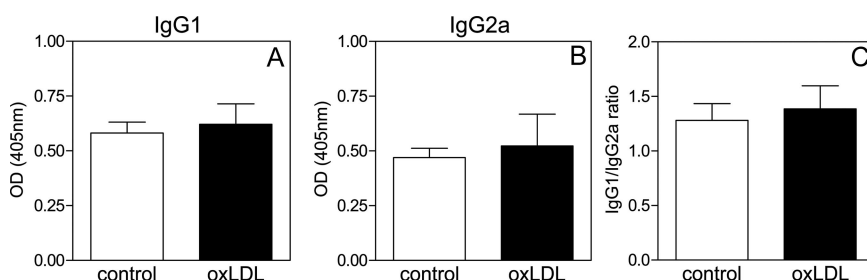


**Figure 7.** Effect of tolerance to oxLDL on oxLDL-specific IgG1 and IgG2a levels. LDLr$^{-/-}$ mice were treated orally with PBS or oxLDL, and serum levels of oxLDL-specific IgG1 (A) and IgG2a (B) were measured with a capture ELISA. Values are mean±SEM. C, Effect of oral tolerance induction to oxLDL on the IgG1/IgG2a ratio.

regulatory T cells can modulate the action of oxLDL-specific CD4^{+} T cells.[42,43] TGF-$\beta$–T$\beta$ RII interaction leads to the activation of a Smad-dependent pathway, resulting in a blockade of IL-2 production[43,44] and a reduced proliferation of oxLDL-specific T cells.

In conclusion, we show that LDLr^{−/−} mice can be tolerized to oxLDL, and that this results in attenuation of both early and advanced atherosclerotic lesions. The mechanism underlying this effect can be dedicated to the induction of CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells, which counteract within the plaque the oxLDL-specific CD4^{+} T cells. These results are promising and prove that the mechanism of oral tolerance induction could be an effective treatment for atherosclerosis.

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## Disclosures

None.

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## CLINICAL PERSPECTIVE

The discovery of the important role of inflammation in both early and advanced stages of atherosclerosis has led to a quest to develop an antiinflammatory therapy for atherosclerosis in patients. Such an antiinflammatory therapy could be more specific and efficient than the present lipid-lowering and blood pressure–lowering therapies. Recent studies have shown that regulatory T cells play an important role in controlling the development of atherosclerosis. Regulatory T cells produce antiinflammatory cytokines such as interleukin-10 and transforming growth factor-$\beta$ and can be induced via tolerance induction. In the present study, we tolerized atherosclerosis-prone mice to oxidized low-density lipoprotein (oxLDL) via 4 oral injections with low doses of oxLDL. This treatment results in a reduction in early (71.2%) and advanced (24.3%) atherosclerotic lesions. The antiatherogenic effect of this therapy is a result of a strong increase in the number of CD4+CD25+Foxp3+ regulatory T cells in the spleen and mesenteric lymph nodes. This increase coincides with increased production of the antiinflammatory cytokine transforming growth factor-$\beta$ and increased mRNA expression of Foxp3, CD25, and CTLA-4 in the atherosclerotic lesions. The beneficial effects of oral tolerance induction to oxLDL on atherosclerosis and the low costs indicate that this may provide new therapeutic approaches for the treatment of atherosclerosis.