Regulation of T cell responses in atherosclerosis

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Chapter 2

Induction of oral tolerance to oxidized LDL ameliorates atherosclerosis

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Abstract
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. Tolerance to oxLDL and malondialdehyde-treated LDL (MDA-LDL) was induced in $LDLr^{-/-}$ 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 (30-71%; $P<0.05$) 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-β 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. In conclusion, oxLDL-specific T cells, present in $LDLr^{-/-}$ mice and important contributors in the immune response leading to the 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 may be a new way to treat atherosclerosis.

Introduction

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 especially Th1 cells producing Th1 cytokines such as interferon- IFN-γ, interleukin (IL)-12, IL-2 and tumor necrosis factor (TNF)-α.[3–5] In addition, immunoglobulin G2a (IgG2a) antibodies against oxLDL, indicating Th1 assistance, predominate in plasma of low-density lipoprotein 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 I diabetes.[12,13] Antigens administered orally enter the gut associated lymphoid tissue (GALT),[14,15] which, as primary function, protects the host from ingested pathogens and proteins.[16,17] Currently, 2 primary effector mechanisms of oral tolerance induction are known. Feeding 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 three groups: Th3 cells and Tr1 cells, mediating suppression via secretion of TGF-β 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-β 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 β2-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-β 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 μM CuSO4 at 37°C for 20 hrs[26] and MDA-LDL was made by addition of 0.5 M MDA to 10 mg of LDL for 3 hours at 37°C.

Immunizations

LDLr$^{-/-}$ mice were immunized with 100 μg of oxLDL or MDA-LDL together with 100 μg of DDA via one i.p. injection. After 14 days spleens were used in proliferation assays.

Spleen cell proliferation assay

Spleen cells were collected by squeezing the spleen through a 70 μm cell strainer, erythrocytes were removed by an erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Splenocytes were cultured at $2{\cdot}10^5$ cells/well in 96-wells plates in RPMI 1640 (2 mM L-Glutamine, 10% FCS) for 24-48 hours. Concanavalin A (Con A; Sigma-Diagnostics, MO) was used as a positive control. Cultures were pulsed for the final 16 hours with [6-^{3}H]-thymidine (1 μCi/well, Amersham Biosciences, The Netherlands). Subsequently, cells were washed, lysed and [^{3}H]-thymidine incorporation was measured. Responses are expressed as stimulation index (SI): ratio of mean counts per minute of triplicate cultures with antigen to triplicate cultures without antigen.

Flow cytometric analysis

Splenocytes were incubated with oxLDL (1-10 μg/ml) in the presence of anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml). 48 hours later cells were harvested, incubated with 1% normal mouse serum and stained with PerCP-conjugated anti-CD3 and FITC-conjugated anti-F4/80 (0.5 μg Ab/200,000 cells). After washing, cells were analyzed by flow cytometry on a FACSCalibur. To detect CD4$^+$CD25$^+$Foxp3$^+$ T cells, a three color flow cytometry was performed. Mononuclear cells were isolated from spleen, mesenteric lymph nodes, Peyer's patches, and blood using Lympholyte (Cedarlane, Canada). Cells were stained

with FITC-conjugated anti-CD4 (0.125 μg/sample) and APC-conjugated anti-CD25 (0.06 μg/sample) mAb, cells were washed, fixed and permeabilized. Subsequently cells were stained with PE-conjugated anti-Foxp3 (0.2 μg/sample). Cells were washed and analyzed by FACS. Data were analyzed with CELLQuest software (BD Biosciences, The Netherlands) and antibodies were from eBioscience, Belgium.

Induction of atherosclerosis

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

Oral tolerance induction during atherosclerosis

To induce tolerance LDLr$^{-/-}$ mice were fasted for 16 hrs. 2 mg of soybean trypsin inhibitor (STI, Sigma, MO) was administered orally to prevent antigen degradation and 10 minutes thereafter mice orally received PBS, 30 μg of Super Oxide Dismutase, (SOD, Sigma-Diagnostics, MO), 30 μg of oxLDL or MDA-LDL. Injections were repeated 3 times to a total of 4 injections in 8 days. After administering the antigens mice were kept on Western-type diet. This was performed either in the first week of diet or after 10 weeks of diet.

Plaque analysis

Six weeks after collar placement the mice were anaesthetized with ketamine-hypnorm and perfused with FormalFixx. Common carotid arteries and both carotid bifurcations were removed for analysis.[27] Arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and 5 μm sections were made on a Leica Cryostat proximal to the collar. Cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). For analysis of the aortic root 10 μm thick sections were made of the aortic root containing the aortic valves. Sections were stained with Oil-Red-O and hematoxylin. Plaque sizes were measured using a Leica DM-RE microscope and LeicaQwin software. Sections were also stained immunohistochemically using antibodies against a macrophage-specific antigen (MOMA-2, Research Diagnostics Inc) and α-smooth muscle cell actin (monoclonal mouse IgG2a, Sigma) exactly as described.[28]

Cytokine assays

Mesenteric lymph node cells were cultured at $2{\cdot}10^6$ cells/ml with or without 5 μg/ml oxLDL. IL-10, IFN-gamma (eBioscience, Belgium) and TGF-β (Bender MedSystems, Austria) concentrations were determined by ELISA in the supernatants.

Real-time PCR assays

Carotid arteries from control and oxLDL-treated mice were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems, CA) using SYBR green technology. The following primer pairs were used: 5′-GGAGCCGCAAGCTAAAAGC-3′ and 5′-TGCCTTCGTGCCCACTGT-3′ for Foxp3; 5′-CTTATATTGCAAATGTGGCACAATC-3′ and 5′-ATCAATCATCAGTGGGACAATCTG-3′ for CD25; 5′-CGAGGTCCTGCACCAACTG-3′ and 5′-TCCATCACCATCGGTTTATGC-3′ for CTLA4. Acidic ribosomal phosphoprotein PO (36B4) was used as the endogenous reference gene and detected using the primers 5′-GGACCCGAGAAGACCTCCTT-3′ and 5′-GCACATCACTCAGAATTTCAATGG-3′.

Detection of anti-oxLDL antibodies

OxLDL (5 μg/ml) dissolved in a $NaHCO_3/Na_2CO_3$ buffer (pH 9.0) was coated. Measurement of IgG1 and IgG2a levels in serum was performed using an ELISA Ig detection kit (Zymed Laboratories, CA) conform the manufacturer's protocol and appropriate controls were performed.

Statistical analysis

All data are expressed as mean$\pm$SEM. The two-tailed student's t-test was used to compare proliferative responses to antigens, FACS data, differences in cytokine production, and atherosclerotic parameters between the different groups.

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 μg/ml, resulted in a 1.53$\pm$0.17 ($P<0.05$) and 2.52$\pm$0.23 ($P<0.001$) fold increase in proliferation, respectively (Figure 2.1A). In case of MDA-LDL, incubation of splenocytes with 1, 5 and 10 μg/ml of MDA-LDL, resulted in a 1.62$\pm$0.15 (ns), 2.90$\pm$1.15 ($P<0.05$) and 4.41$\pm$0.89 ($P<0.01$) fold increase in proliferation, respectively (Figure 2.1B). OxLDL was toxic at concentrations $>$10 μg/ml, whereas MDA-LDL was only toxic at concentrations $>$100 μg/ml (data not shown). In all experiments conA, 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 μg 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 μg/ml of 2.89±0.29 and a 7.25±0.81 fold, respectively, compared with the controls (P<0.01) (Figure 2.1C). Incubation of splenocytes isolated from MDA-LDL immunized mice with 1, 5 and 10 μg/ml MDA-LDL resulted in a 2.07±0.82 (ns), 3.94±0.41 (P<0.01) and 6.17±1.50 (P<0.05) fold increase in proliferation (Figure 2.1D).
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±1.5% to 46.9±2.4% when splenocytes were incubated with 5 μg/ml of oxLDL (Figure 2.1E; P<0.05). The amount of macrophages was not affected by the incubation with oxLDL (data not shown).

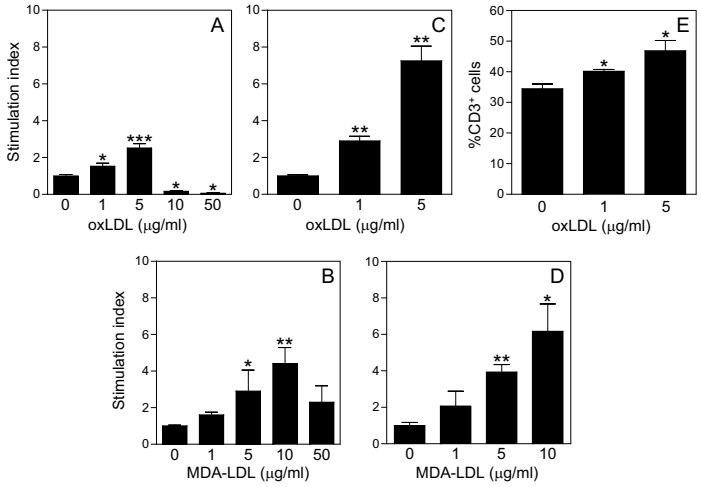

Figure 2.1: Spleen cell proliferation in response to oxLDL and MDA-LDL. $LDLr^{-/-}$ mice were immunized via one i.p. injection with 100 μg of oxLDL or MDA-LDL. Two weeks thereafter, splenocytes were isolated from naive and immunized $LDLr^{-/-}$ mice and cultured with oxLDL (A and C, respectively) or MDA-LDL (B and D, respectively) for 24 hrs. In the control situation, splenocytes from naive or immunized mice were cultured in 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 (S.I.) ± SEM. The S.I. 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. Graph E shows the percentage of $CD3^+$ cells (mean±SEM) after proliferation of the splenocytes with 1, 5 and 10 μg/ml of oxLDL. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001

Effect of oral tolerance induction to oxLDL and MDA-LDL on the 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 one week and subsequently, oral tolerance was induced by oral administration of 30 μg of oxLDL or MDA-LDL. Treatment was repeated every other day to four times in total. After tolerance induction, the Western-type diet feeding was continued and one-week later mice were equipped with collars around both carotid arteries to induce atherosclerosis.[29] During the experiment

total plasma cholesterol levels were not significantly different between the groups and increased from 679±64 mg/dl (before diet) to 1554±140 mg/dl in control mice, to 1755±146 mg/dl in oxLDL treated mice and to 1718±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 2.2A, 2.2B). Oral feeding of $LDLr^{-/-}$ mice with SOD had no effect on plaque size as compared to PBS (data not shown). Oral tolerance induction to oxLDL resulted in a significant 71.2% reduction in plaque area (Figure 2.2C; 20768±5964 versus 6046±1941 μm^2; $P<0.05$) when compared to control mice. Furthermore, a beneficial 55.3% reduction in intima/lumen ratio (Figure 2.2D; 0.25±0.06 versus 0.11±0.03; $P<0.05$) and a 72.1% reduction in intima/media ratio (data not shown; 0.75±0.23 versus 0.21±0.04; $P<0.05$) was observed. In case of MDA-LDL, oral tolerance induction did not result in a significant effect on plaque size (Figure 2.2E; 23314±8016 versus 37327±13137 μm^2; P=0.41) and also no significant effect on intima/lumen ratio (Figure 2.2F; 0.29±0.12 versus 0.50±0.16; P=0.34) and intima/media ratio (data not shown; 0.42±0.15 versus 1.12±0.34 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 2.3B) resulted in a significant 29.9% reduction in plaque size (Figure 2.3C; 669243±56643 versus 469288±27950 μm^2; $P<0.01$) when compared with control-treated mice (Figure 2.3A). 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 when compared to 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 μg 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±67 to 2727±243 mg/dl and 2422±366 mg/dl in the control and oxLDL-treated mice, respectively (no significant difference). After 7 weeks, the mice were sacrificed, cryosections of the aortic root of control-treated (Figure 2.4A) and oxLDL-treated (Figure 2.4B) 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 2.4C; 715523±56365 μm^2 versus 541353±55239 μm^2; $P<0.05$) as compared to control-treated mice. The effect of oral tolerance induction to oxLDL is more impressive when it is taken into account that the size of the plaques at the time of tolerance induction was 300000 μm^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 2.4D). Interestingly, no effect of tolerance induction to oxLDL was observed on the relative macrophage and

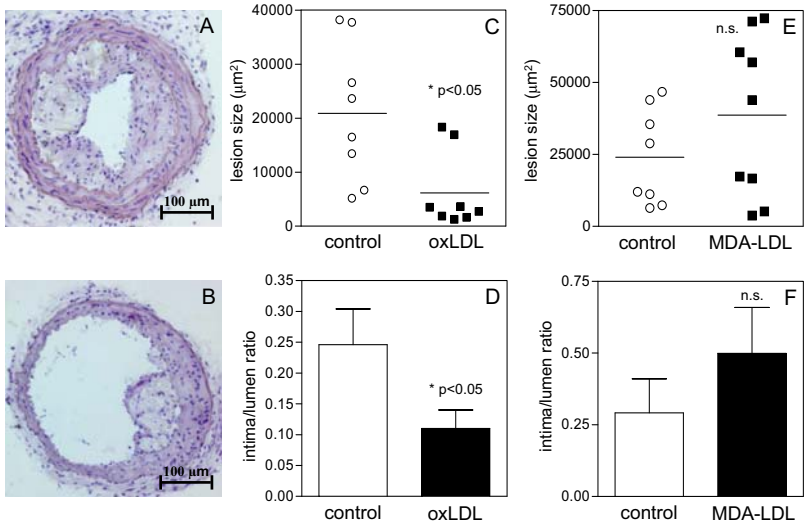

Figure 2.2: Effect of oral tolerance induction to oxLDL on atherosclerotic plaque formation in collar induced atherosclerosis in the carotid artery LDLr$^{-/-}$ mice were fed oxLDL or MDA-LDL four times before atherosclerosis was induced by collar placement around both carotid arteries. Six weeks after 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. $^{*}P<0.05$

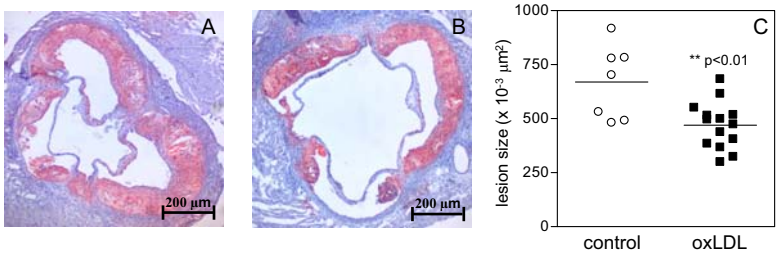

Figure 2.3: Effect of oral tolerance induction to oxLDL on atherosclerotic plaque formation in the aortic root LDLr$^{-/-}$ mice were fed oxLDL four times before atherosclerosis was induced by feeding a Western-type diet. After eight weeks of diet mice were killed and the aortic roots of control-treated (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). $^{**}P<0.01$

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±0.2%), mesenteric lymph nodes (3.0±0.4%), Peyer's patches (1.7±0.4%) and blood (1.7±0.1%) of control LDLr$^{-/-}$ mice. Two and four days after the

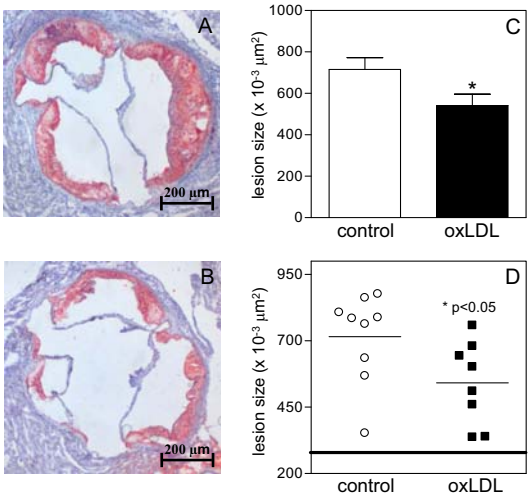

Figure 2.4: Oral tolerance induction to oxLDL attenuates atherosclerotic plaque progression in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks before oxLDL was administered orally 4 times. After oral tolerance induction the mice were kept on a Western-type diet for 7 more 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. Graph D shows a dot plot of lesion size in all mice. The bar at $0.30{\cdot}10^6$ μm^2 represents the lesion size of mice after 10 weeks of diet without oral treatment. $^*P<0.05$

fourth and last oral feeding of oxLDL, the number of CD4$^+$CD25$^+$Foxp3$^+$ cells increased significantly to 1.3±0.1% and 1.6±0.2% in the spleen, respectively (Figure 2.5A; $P<0.05$) and to 5.2±0.2% and 5.8±0.6% in mesenteric lymph nodes, respectively (Figure 2.5B; $P<0.01$). No significant changes were seen in the Peyer's patches and blood. To determine if the effect was long lasting, mice were sacrificed 14 days after the last oral feeding. CD4$^+$CD25$^+$Foxp3$^+$ cells were still increased in spleen and mesenteric lymph nodes (1.4±0.1% ($P<0.05$) and 5.4±0.3% ($P<0.01$), respectively) after oral feeding of oxLDL (Figure 2.5A, 2.5B), 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 production, mesenteric lymph nodes of control, oxLDL, and MDA-LDL-treated mice were re-stimulated with 5 μg/ml oxLDL or MDA-LDL *in vitro*. We observed that oxLDL only induced a significant 6-fold increase in TGF-β in lymph node cells from oxLDL tolerant mice, but not in control pretreated mice, whereas MDA-LDL was unable to induce TGF-β in MDA-LDL pretreated or control mice (not shown). The IL-10 and IFN-γ levels were in any of the experiments below the detection limits.

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 when compared with

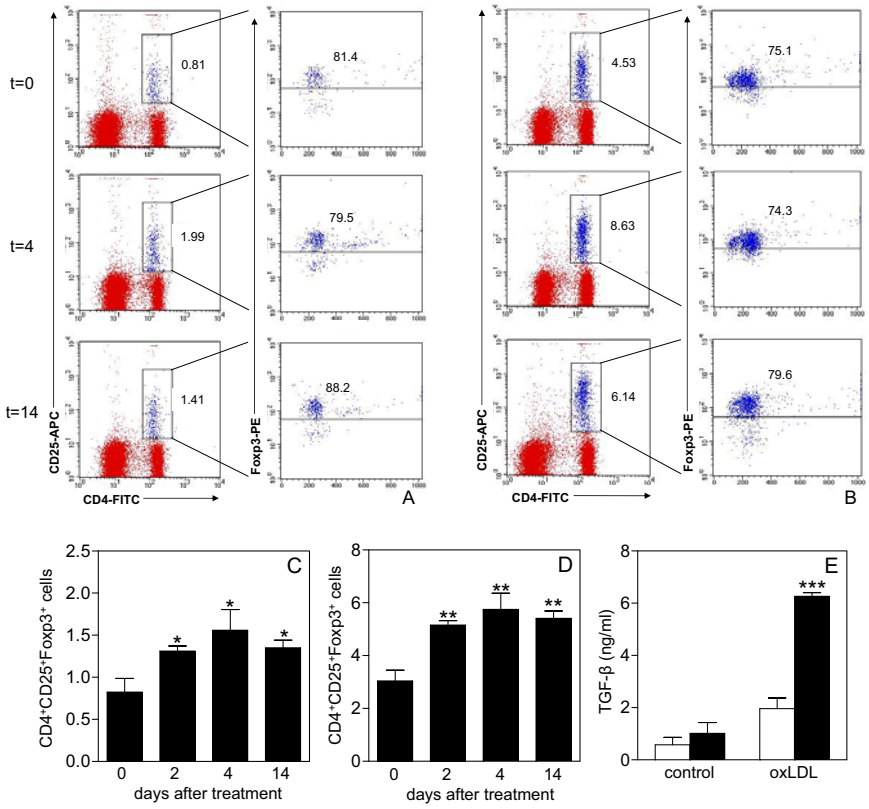

Figure 2.5: Effect of oral tolerance induction to oxLDL on $CD4^+CD25^+Foxp3^+$ cells in spleen and MLNs. $LDLr^{-/-}$ mice were fed PBS or oxLDL 4 times and were killed at day 2, 4 and 14. The dot plots show representative examples of lymphoid cells isolated from the spleen (A) and mesenteric lymph nodes (B) stained for CD4 and CD25 (left panels). The right panel of dot plots shows the percentage of $Foxp3^+$ cells within the $CD4^+CD25^+$ population. Graph C represents the percentage of $CD4^+CD25^+Foxp3^+$ cells in the spleen, graph D the percentage of $CD4^+CD25^+Foxp3^+$ cells in the mesenteric lymph nodes (mean±SEM). (E) TGF-β 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*. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (compared to all other bars)

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 2.6; $P<0.05$).

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 the initiation of atherosclerosis. No detectable differences in IgG1 and IgG2a levels were observed (Figure 2.7A, 2.7B, respectively) and no difference in the IgG1/IgG2a ratio in control and oxLDL-treated mice was observed (Figure 2.7C).

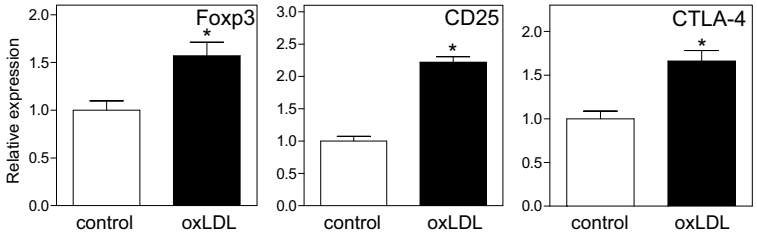

Figure 2.6: Expression of regulatory T cell markers in oxLDL-treated and control mice. mRNA was isolated from carotid arteries of control (n=8) and oxLDL-treated (n=14) mice and the mRNA levels of Foxp3, CTLA-4 and CD25 were quantitatively determined and expressed relative to 36B4. $^{*}P<0.05$

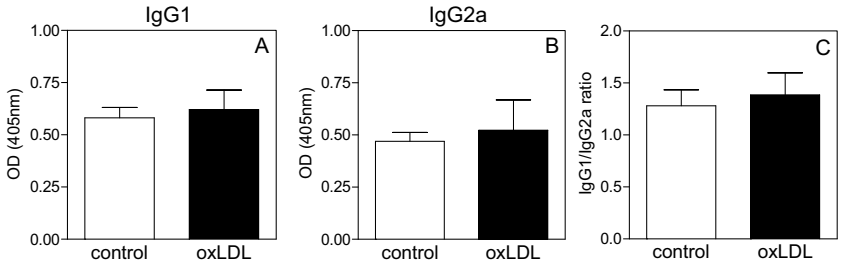

Figure 2.7: Effect of tolerance to oxLDL on oxLDL-specific IgG1 and IgG2a levels. LDLr$^{-/-}$ mice were treated orally with PBS or oxLDL and serum levels of oxLDL-specific IgG1 (A) and IgG2a (B) were measured using a capture enzyme-linked immunosorbent assay. Values are mean OD(405) values $\pm$ SEM. Graph C represents the effect of oral tolerance induction to oxLDL on the IgG1/IgG2a ratio.

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 apolipoprotein B-100 peptides,[30] and oxidized phosphorylcholine-containing phospholipids,[31] can be presented by antigen presenting cells and result in T cell activation.[32] 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 (3-7-fold) in the immunized mice when compared to 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 via Th1 cytokine inhibition the extent of atherosclerosis can be reduced.[28,33–35] The other way around, stimulation of the Th2 cytokine production can attenuate atherosclerosis.[29,36] Although restoration of the imbalance between Th1 cells and Th2 cells may be effective in 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 pro-atherogenic.[33] Mallat et al. hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and/or Th2) and regulatory T cells (Tregs) specific for 'altered' self and non-self antigens.[37] Recently, Ait-Oufella et al. showed that regulatory T cells play an important role in controlling the development of atherosclerosis.[38] 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 $CD4^+CD25^+Foxp3^+$ 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-β and CTLA-4.[21]

In our current study we show that oral tolerance induction to oxLDL can both attenuate atherosclerosis in an early stage and in an advanced stage. A relative low dose of oxLDL (30 μg, four 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 results are in line with studies that showed that oral or nasal tolerance induction to HSP65 and β2-glycoprotein I may be a useful treatment for atherosclerosis.[22–24] In our current study also a significant 24.3% reduction in advanced atherosclerotic lesion size was observed. Taken the initial lesion size into account a 42.4% reduction in plaque progression is obtained. In line with the studies on HSP65 and β2-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 overexpression 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 SMC content of the plaque.[29]

Besides oxLDL, MDA-LDL was also 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 μg 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 also 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 demonstrate 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. Increasing levels of IL-10 and TGF-β after oral tolerance induction are dedicated to a stimulated development of adaptive immune cells.[24] Zhang et al. found an increased amount of $CD4^+CD25^+$ cells after feeding mice with

OVA. These "regulatory" T cells were declared to be responsible for the high levels of IL-10 and TGF-β.[41] Recently, $CD4^+CD25^+$ cells with Foxp3 expression were found to be immuno-suppressive. We demonstrate that oxLDL tolerance induction significantly increases the number of $CD4^+CD25^+Foxp3^+$ cells for up to two weeks in the spleen and the mesenteric lymph nodes. Since these regulatory T cells exert their suppressive effect via cell-cell contact and surface-bound TGF-β, we determined the TGF-β production in the mesenteric lymph nodes. Lymph node cells from oxLDL tolerant mice produced 6-fold higher levels of TGF-β upon re-stimulation with oxLDL than cells from control mice, indicating the induction of TGF-β 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 upon tolerance induction. The profile of gene expression upon oxLDL tolerance induction, together with the induction of Foxp3 in spleen and lymph nodes and concurrent oxLDL specific TGF-β production in the absence of IL-10 and IFN-γ production highly suggest that $CD4^+CD25^+Foxp3^+$ Tregs rather than Tr1 or Th3 are responsible for the observed effects of oxLDL tolerance induction on atherosclerosis. The absence of induction of Tregs by MDA-LDL and the absence of TGF-β induction in lymph node cells of MDA-LDL pretreated cells by MDA-LDL may explain the absence of an regulatory effect of oral treatment with MDA-LDL.
It may be speculated that oxLDL specific Tregs 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 within the plaque activated by oxLDL presenting APCs, which may lead to the expression of TGF-β receptor II (TβRII) by these cells. OxLDL specific Tregs also recognize oxLDL presented by the APCs and via the enhanced production of TGF-β, Tregs can modulate the action of oxLDL specific $CD4^+$ T cells.[42,43] TGF-β-TβRII 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 describe that $LDLr^{-/-}$ mice can be tolerized to oxLDL which results in an attenuation of both early an 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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