The potential use of dendritic cells in mouse models of atherosclerosis

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Chapter 2

Vaccination using oxLDL-pulsed DCs reduces atherosclerosis in LDLr$^{-/-}$mice

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Abstract

Modification of lipoproteins plays an important role in the development of atherosclerosis. Oxidatively modified low-density lipoprotein (oxLDL) has a number of pro-inflammatory effects, whereas immunization with various forms of oxLDL is able to reduce atherosclerosis. The uptake of modified LDL by dendritic cells (DCs) and the presentation of epitopes thereof may form an important step in the immunomodulatory effects of LDL. In this study we transferred oxLDL-pulsed mature DCs (mDCs) to $LDLr^{-/-}$ mice and examined the effects on atherosclerosis. Bone-marrow derived DCs were cultured for 10 days in the presence of granulocyte-macrophage colony-stimulating factor. Immature DCs were matured by lipopolysaccharide and pulsed with copper oxidized LDL. These DCs were transferred three times to $LDLr^{-/-}$ mice before the induction of atherosclerosis by Western-type diet feeding. Transfer of oxLDL-pulsed mDCs resulted in an 87 % reduction in carotid artery lesion size ($P<0.01$) with a concurrent increase in plaque stability while treatment with mDCs pulsed with the atherosclerosis irrelevant antigen, ovalbumin, did not influence lesion size or stability. Furthermore, the vaccination procedure resulted in the induction of oxLDL-specific T cells with a reduced Th1 profile and an increase in oxLDL-specific IgG levels, which contributed to a reduction in foam cell formation. In conclusion, these data indicate that vaccination with oxLDL-pulsed mDCs provides a novel and powerful strategy for the immunomodulation of atherosclerosis.

Introduction

Atherosclerosis is a slowly progressing disease that develops at sites of lipid accumulation in large and medium sized arteries, which can lead to infarction of the heart or the brain. Over the past several years, accumulating data has identified a key role for inflammation in atherosclerosis and both innate and adaptive immune responses are involved.[1-5] Several antigens have been implicated in the initiation of immune responses during atherosclerosis including exogenous infectious pathogens such as *Chlamydia pneumoniae*, and cytomegalovirus but also endogenous proteins such as heat-shock proteins and β2-glycoprotein-Ib.[6-8] The most intensively studied endogenous antigen is oxidized low-density lipoprotein (oxLDL). Oxidation of lipoproteins in the arterial intima, followed by their uptake by macrophages and subsequent foam cell formation, plays an important role in the development of atherosclerosis. In addition, oxidation of LDL results in many structural modifications of apoB-100 and thus the formation of many neo-epitopes, which renders the modified LDL immunogenic and leads to both a cellular and humoral response. Since the different epitopes of oxLDL induce atherogenic immune responses, it is attractive to modulate the immune response towards oxLDL. Also, a number of studies show that immunization against oxLDL reduces atherosclerosis in several animal models.[9-11]

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system.[12-14] Immature DCs differentiate from bone-marrow progenitors or circulating blood monocytes. They reside in the blood stream or peripheral tissues where they survey incoming pathogens. An interaction with pathogens induces maturation during which DCs generate MHC-peptide complexes and upregulate the expression of costimulatory molecules such as CD40, CD80 and CD86. These changes render the DCs fully competent to activate T cells. Several studies showed that oxLDL induces several changes, characteristic for DC maturation, including a higher expression of costimulatory molecules and the increased ability to stimulate T cells. [15, 16]

Due to their potent capacity to stimulate T cells, DCs are being investigated in vaccine and therapy approaches.[17, 18] We aimed to assess the use of oxLDL-pulsed mature DCs (mDCs) as an immunotherapy for atherosclerosis. DCs obtained from the bone-marrow can be pulsed *ex vivo* by inducing maturation in the presence of oxLDL. In this study we show that oxLDL-pulsed mDCs induce oxLDL-specific T cells, a lowered Th1-response and an increased production of oxLDL-specific antibodies and this accumulates in a reduction in lesion size. In conclusion, these data indicate that vaccination with oxLDL-pulsed mDCs triggers the immune system against oxLDL, which proves to be beneficial for the treatment of initial phases of atherosclerosis.

Materials and methods

Media and reagents

Cell culture medium for the dendritic cells was IMDM (Cambrex, Belgium) supplemented with 8 % FCS (PAA, Germany), 100 U/ml streptomycin/penicillin (PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μM β-mercaptoethanol. LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave *et al*.[19] The isolated LDL was dialyzed against phosphate buffered saline (PBS) with 10 μM EDTA (pH 7.4) for 24 hours at 4°C and oxidized by exposure to 10 μM $CuSO_4$ at 37°C for 20 hours as previously described.[20] For the proliferation studies and the induction of foam cells we used RPMI 1640 medium supplemented with 2 mM L-Glutamine, 10% FCS and 100 U/ml streptomycin/penicillin (PAA, Germany) (cRPMI). For the culturing of Bone-marrow derived macrophages cRPMI with 20% FCS was used.

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr-/- mice and UBC-GFP mice were from Jackson Laboratories on a C57Bl6 background and bred in-house. Male C57BL/6J mice were from Charles River Laboratories. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 10-12 weeks old at the start of the experiment. Diet and water were administered ad libitum.

Generation and injection of BM-DCs

For each injection time-point, bone marrow cells were isolated from the tibia and femora of 3 C57BL/6 mice. Cells were immediately pooled and cultured during 10 days in complete IMDM in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). After 10 days of culture, immature DCs were activated with 1 μg/ml of lipopolysaccharide (LPS from Salmonella typhosa, Sigma Aldrich, Zwijndrecht, The Netherlands) during 24 hours. Simultaneously, DCs were pulsed with or without 7.5 μg/ml of copper-oxidized LDL or 1 mg/ml of ovalbumin. Purity and functionality of the DCs were assessed using flow cytometry. CD11c was used as a specific DC marker (purity > 90%). Functionality was determined by the expression of several costimulatory molecules (CD40, CD80, CD86) and the expression of MHC-II and CD1d. Mice were injected intravenously (8, 6 and 3 days prior to atherosclerosis induction by Western-type diet feeding) with 200 μl of PBS (n = 8) or 1.5 x 10^6 DCs (oxLDL-pulsed or unpulsed mDCs) in 200 μl PBS (n = 11). DCs from the UBC-GFP mice were identically isolated and cultured.

Assessment of atherosclerosis

Male $LDLr^{-/-}$ mice were injected 3 times intravenously (1 injection every other day) with 1.5 x10^6 DCs or saline prior to Western-type diet feeding. After 3 weeks of diet, atherosclerosis was induced by bilateral perivascular collar placement (2 mm

long, diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding.[21] Seven weeks after collar placement, mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS and subsequent perfusion using formalin. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use.

Histological analysis and morphometry

Transverse cryosections were prepared in a proximal direction from the carotid bifurcation. Cryosections were stained with hematoxylin (Sigma Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica, Germany) or with hematoxylin and Oil-Red-O. Hematoxylin-eosin stained sections of right carotid artery (5 µm) and Oil-Red-O stained sections of heart valves (10 µm) were used for morphometric analysis of atherosclerotic lesions as described previously.[21] Corresponding sections were stained immunohistochemically with antibodies against a macrophage specific antigen, (MOMA-2, polyclonal rat IgG2b, Research Diagnostics Inc, NJ) or were stained for collagen fibers using the Masson's Trichrome method (Sigma Aldrich, Zwijndrecht, The Netherlands). The images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK).

Proliferation assay

Spleens from mice treated with oxLDL-pulsed mDCs (n=5) or mDCs (n=5) were isolated and smashed through a 70 µm filter. Red blood cells were lysed using 0.83% NH_4Cl in 0.01 M tris/HCL, pH 7.2. Splenocytes were cultured in triplicates at $2x10^5$ cells/well in the presence or absence of oxLDL (5 µg/ml) in complete RPMI 1640 (cRPMI). Concanavalin A (ConA, Sigma-Diagnostics, MO) (2 µg/ml) was used as a positive control. After 24 hours cytokine levels were determined in the supernatant according to the manufacturer's protocol (Mouse Th1/Th2 ELISA, Ebioscience, Belgium). Parallel cultures were pulsed for an additional 16 hours with 1 µCi/well of ^{3}H-thymidine (Amersham Biosciences, The Netherlands) and the amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R) as the number of disintegrations per minute (DPM).

Foam cell formation

To obtain macrophages, bone-marrow cells from C57BL/6 mice were resuspended in complete RPMI supplemented with 20% FCS and 30% of L929-conditioned medium (source for monocyte-colony-stimulating factor, M-CSF). After 7 days of culture, macrophages were seeded in Lab-Tek chamber slides (BD Falcon, The Netherlands) ($0.8 x10^6$ cells/well) in cRPMI. Cells were starved for 4 hours after which a mixture of oxLDL (10 µg/ml) + an equal volume of mouse serum (2 fold diluted, sera from 2 mice were pooled) was added. This mixture of oxLDL and mouse serum was made 30 minutes before addition to the cells. After overnight culture, cells were fixed using zinc formal Fixx, stained for lipids using Oil-Red-O and counter stained with hematoxylin. The amount of Oil-Red-O staining was corrected for the number of cells as indicated by the hematoxylin staining. 8 random fields per condition were analyzed using the Leica DM-RE microscope and

LeicaQwin software.

Cholesterol assay
Blood was collected at several time points during the experiment by tail vein bleeding. Plasma was obtained after centrifugation and total plasma cholesterol levels were measured using enzymatic procedures and a spectrophotometer (Roche Diagnostics, The Netherlands). Precipath was used as an internal standard (Boehringer, Mannheim, Germany). The cholesterol distribution over the different lipoproteins was analyzed by fractionation using a Superose 6 column (3.2 x 30 mm, Smart-System, Pharmacia).

Cytospin
After 24 hours of incubation in the presence of oxLDL, DCs were centrifuged for 5 min at 500 rpm using the Thermo Shandon Cytospin 4. After centrifugation, slides were fixed for 30 minutes using zinc formal Fixx (Shandon, Pittsburgh, USA). Lipid loading in the DCs was visualised using Oil-Red-O staining.

Flow Cytometry
After the culture period, dendritic cells or macrophages were stained with the appropriate Ab (CD11c, CD11b, F4/80, MHC-II, CD40, CD80, CD86 and CD1d, all obtained form Ebioscience, Belgium) to check purity or the maturation status.
After sacrificing the mice, blood, spleen, liver and lymph nodes were isolated (n=5 per group). Single cell suspensions were obtained by smashing the cells through a 70 µm cell strainer (Falcon, The Netherlands). Blood and spleen cells were lysed using 0.83% NH_4CL in 0.01 M Tris/HCL pH 7.2. Mononuclear cells from the liver were isolated from non-parenchymal cells using Lympholyte (Cederlane, Canada). Subsequently 300.000 cells were stained with antibodies (Ebioscience, Belgium) in the presence of 1 % mouse serum. For the detection of subclasses specific Ab, spleen cells were treated as stated above and stained with IgG1 and IgG2c (BD Bioscience, Belgium). FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software.

ELISA for IgM and IgG antibodies against LDL, MDA-LDL and Cuox-LDL
Antibodies against LDL, MDA-LDL and Cuox-LDL were determined by ELISA. MaxiSorp 96 well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 µg native LDL or oxLDL in 100 µl PBS at 4°C. Plates were washed 5 times with 0.01M Tris, 0.15 M NaCl and 0.05% Tween20 (pH 8.0). Mouse serum was added in duplicate at a 1:50 dilution in incubation buffer (0.1 M Tris, 0.3 M NaCl and 0.05% Tween20 (pH 8.0) overnight at 4°C. After washing, plates were incubated with either alkaline phosphatase-labelled anti-mouse IgM or IgG (Jackson Immuno-Research, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. After washing, substrate (1 mg/ml disodium p-nitrophenyl phosphate, Sigma, The Netherlands) was added. After 2 hours at room temperature, absorbance was read at 405 nm.

ELISA for detecting subclasses specific antibodies

IgG1 and IgG2c levels were detected using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Francisco, USA) according to manufacturer's instructions. Briefly, high binding plates were coated overnight with either Cuox-LDL (5 µg/ml) or ovalbumin (10 µg/ml). Serum samples were 1:1 diluted in PBS. Absorbance was read at 405 nm.

ELISA for detecting cytokines

Levels of IFN-γ, IL-10 and IL-4 were measured in the supernatants of the ConA stimulated spleen using the Th1/Th2 Ready-Set-Go Kit (Ebioscience, Belgium). Detection limits are 15 pg/ml; 30 pg/ml and 4 pg/ml, respectively. In short, triplo cultures from the spleen cells treated with ConA were pooled and cytokine levels were determined in the undiluted supernatant following the manufacturer's instructions.

Statistical analysis

Values are expressed as mean ± SEM. Data were analyzed with either a parametric or non-parametric ANOVA when comparing three groups or a two-tailed Student's t-test or the Mann-Withey U test when comparing two groups. Statistical analysis was performed using the Instat3 software. Probability values of $P<0.05$ were considered significant.

Results

Effect of oxLDL on dendritic cells

We investigated whether DCs engulfed oxLDL and if the uptake of lipoproteins affected the maturation of DCs. Increasing concentrations of oxLDL (up to 20 µg/ml) were added to immature DCs. After 24 hours of incubation we stained the intracellular lipid accumulation in the DCs with Oil-Red-O and analyzed for surface markers by flow cytometry. We found that increasing oxLDL concentrations induced an increasing lipid accumulation, but oxLDL levels exceeding 7.5 µg/ml induced a concentration dependent increase in cell death and a lowering of the expression of the DC-specific markers (data not shown). Therefore we chose a concentration of 7.5 µg/ml which induced optimal lipid loading and minimal cell death. The addition of oxLDL to both imDCs and mDCs resulted in lipid accumulation in more than 95% of the DCs (Figure 1B and 1C) compared to the immature control incubated DCs (Figure 1A). During maturation, DCs upregulate the expression of costimulatory molecules and the maturation level of DCs can thus be followed by flow cytometry for several specific markers (CD40, CD80, CD86, MHC-II, CD1d and CCR7). We treated immature DCs with the TLR4 agonist, LPS (1 µg/ml) in the absence or presence of oxLDL (7.5 µg/ml). Treatment with LPS resulted in a typical mature DC cell surface phenotype showing high expression of CD11c (a specific marker for mouse DCs), CCR7 (necessary for migration towards lymphoid organs) MHC-II, CD1d and the costimulatory molecules CD40, CD80, CD86. The addition of oxLDL during maturation had no effect on the expression level of these molecules and thus did not affect the maturation state of the DCs (Figure 1D).

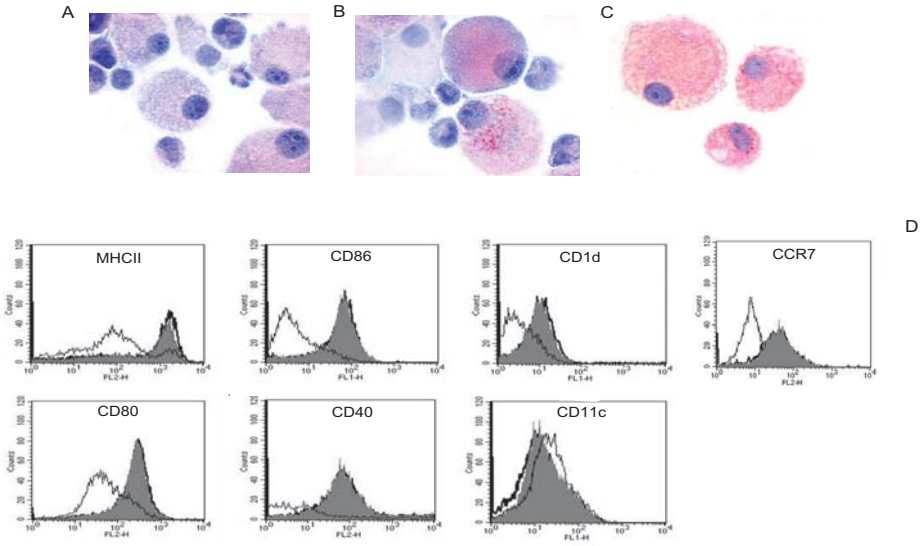

Figure 1: The effect of oxLDL on dendritic cells
Immature DCs were treated with LPS (1 µg/ml) in the absence or presence of oxLDL (7.5 µg/ml). After 24 hours, DCs were collected. Oil-Red-O staining of DCs: immature DCs in the absence of oxLDL (A), in the presence of oxLDL (B) and mature DCs in the presence of oxLDL (C). Figure D: Expression levels of surface markers analyzed by flow cytometry on immature DCs (dotted line), mature DCs (thick line) and mature oxLDL-pulsed DCs (grey).

Dendritic cells home towards lymphoid organs after *i.v.* injection

To determine the fate of DCs *in vivo*, we cultured DCs from UBC-GFP mice, which have a high and constitutive expression of GFP, and injected 1.5 x 10^6 DCs into $LDLr^{-/-}$ mice. We injected both mature unpulsed and mature oxLDL-pulsed GFP-DCs. 72 hours after injection, mice were sacrificed and GFP expression was determined in different tissues using flow cytometry. While there were almost no GFP^+ DCs present in the circulation, DCs did migrate towards the lung and liver, and towards lymphoid organs such as spleen, mediastinal and inguinal lymph nodes (Figure 2). Mature unpulsed and mature oxLDL-pulsed DCs displayed an identical distribution pattern.

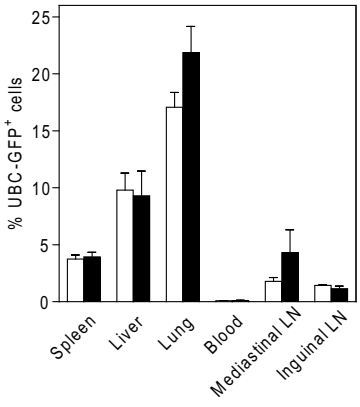

Figure 2: Homing of GFP^+-DCs
1.5 x 10^6 mature GFP^+-DCs (white bars, n=4) and mature oxLDL-pulsed GFP^+-DCs (black bars, n=4) were injected *i.v.* into $LDLr^{-/-}$ mice. Mice were sacrificed after 72 hours. The percentage of GFP^+ cells in different organs are shown.

Vaccination using oxLDL-pulsed mDCs attenuates atherosclerotic lesion formation

To evaluate the effect of vaccination using oxLDL-pulsed mDCs on de novo atherosclerosis, LDLr$^{-/-}$ mice were injected intravenously 3 times (day –8, –6 and –3) with either PBS, mDCs, or oxLDL-pulsed mDCs. On day 0, mice were put on a Western-type diet. Three weeks thereafter, atherosclerosis was induced by perivascular collar placement around the carotid arteries as described[21] and Western-type diet was continued for seven weeks where after mice were sacrificed. Plaque formation in the carotid artery and the aortic root was analyzed using the hematoxylin/eosin staining and hematoxylin/Oil-Red-O staining, respectively. Plaque sizes in mice treated with PBS did not significantly differ from the plaque sizes upon treatment with mDCs. In addition cholesterol levels and morphology of the plaque (see below) were also identical in these groups. Figure 3A-C shows representative examples of hematoxylin/eosin staining in the carotid arteries. Injection of oxLDL-pulsed DCs resulted in a 87 % reduction in plaque size in the carotid arteries (PBS: 48,578±9,231 μm^2; mDCs: 31,919±7,914 μm^2; mDCs+oxLDL: 4,023±504,1 μm^2; P_{anova}=0.0009) (Figure 3D). Also a concomitant 85 % reduction in intima/lumen ratio (PBS: 0.560±0.097; mDCs: 0.461±0.089; mDCs+oxLDL: 0.071±0.009; P_{anova}=0.0003) was observed (Figure 3E). In the aortic root the treatment with oxLDL-pulsed DCs induced a non-significant 24% decrease in plaque size (PBS: 228,667±32,747 μm^2; mDCs: 293,181±36,193 μm^2; mDCs+oxLDL 224,983±48,546 μm^2) (Figure 3F).

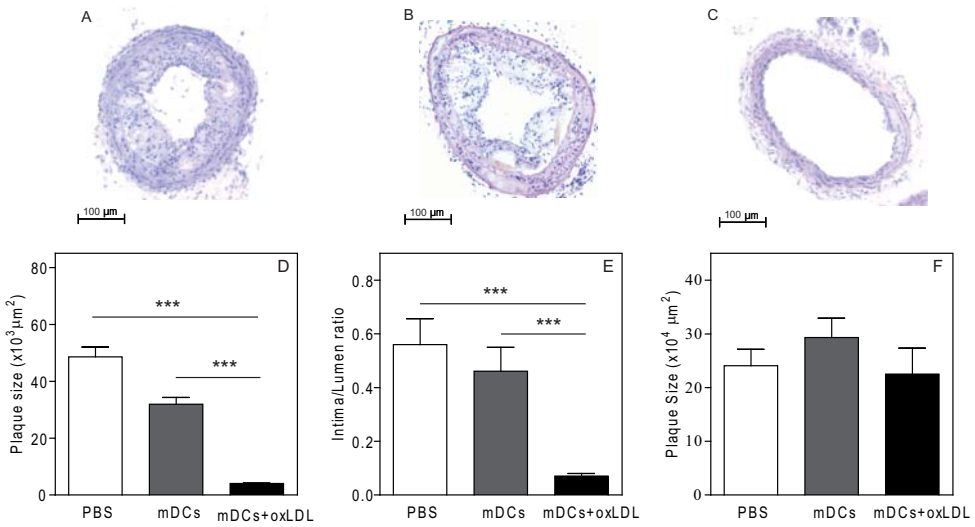

Figure 3: Effect of vaccination on atherosclerosis

Representative sections of lesions in the carotid arteries of PBS, (A; n=9), mDCs (B; n=11) and oxLDL-pulsed mDCs (C; n=11) treated mice. Lesion size (D) and intima/lumen ratio (E) in the carotid arteries were determined. Plaque size in the aortic root was quantified (F)(***P<0.001).

Plaque morphology was assessed by the number of macrophages and the presence of collagen fibers. When comparing mice in the oxLDL-pulsed mDCs group to mice in the mDCs group, we observed a 50% reduction in the MOMA-2/Intima ratio in both the carotid arteries (Figure 4A) and the aortic root (Figure 4D), while the collagen content of the intima was increased in both the carotid arteries (P_{anova}=0.0145) (Figure 4B) and the aortic root (Figure 4E). In addition, we calculated the MOMA-2/collagen ratio as an indicator for plaque stability. A significant 3.9- and 2.8-fold reduction was observed in both the carotid arteries (P_{anova}=0.05) and aortic root, respectively (P_{anova}=0.0097) (Figure 4C and 4F). This reduced MOMA-2/collagen ratio indicates a more stable plaque phenotype in the mice treated with oxLDL-pulsed DCs in both the carotid arteries and the aortic root.

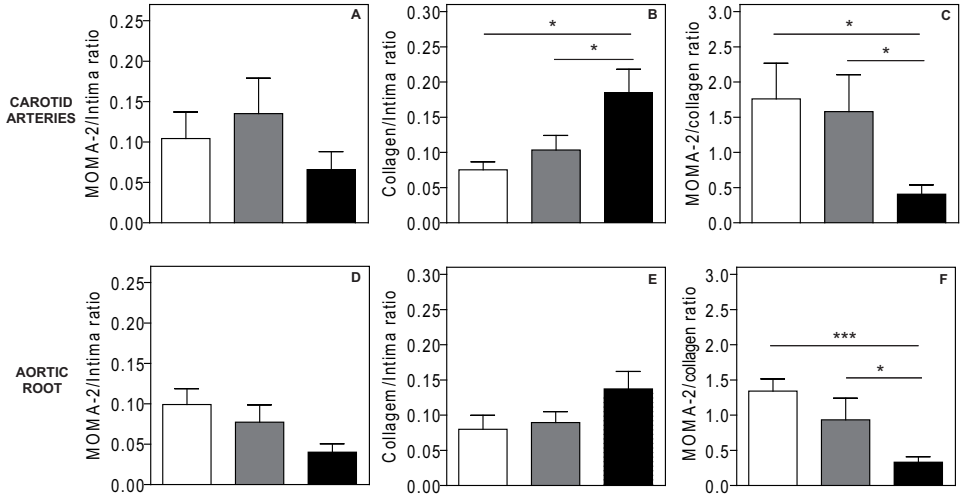

Figure 4: Plaque morphology
Plaque composition was assessed in the carotid artery (A-C) and in the aortic root (D-F). The amount of macrophages was determined as the MOMA-2 positive area per intima area, the amount of collagen was determined by quantifying the blue collagen staining of the Masson's Trichrome stain within the plaque (*P<0.05; ***P<0.001).

Since treatment with mDCs induced a slight, non-significant reduction in atherosclerosis, we additionally tested whether treatment with DCs pulsed with an irrelevant antigen affected atherosclerosis. To that end we treated mice with ovalbumin-pulsed DCs next to oxLDL-pulsed DCs and PBS treatment. Treatment with ovalbumin-pulsed DCs had no effect on plaque size in the carotid artery nor in the aortic root (Figure 5), while it also did not affect plaque composition at these sites (Figure 6). In contrast, oxLDL-pulsed DCs did lower the plaque size in the carotid artery. In addition, the macrophage/collagen ratio in the lesions in both the carotid artery and the aortic root were beneficially lowered in oxLDL-pulsed mDCs treated animals.

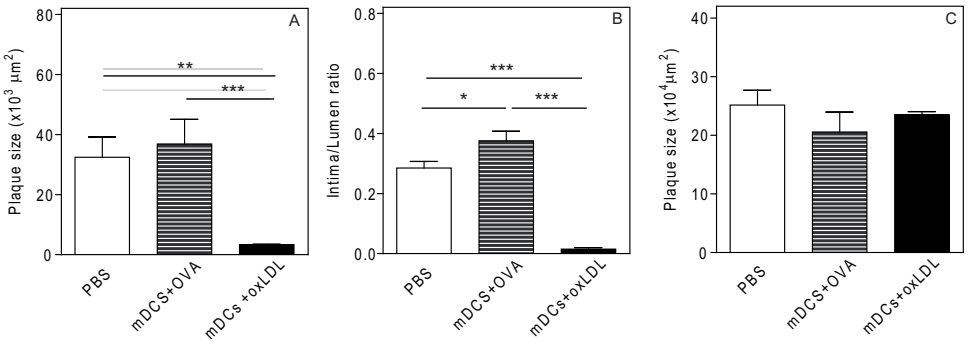

Figure 5: Effect of antigen-pulsed dendritic cells on lesion formation
Lesion size (A) and intima/lumen ratio (B) in the carotid arteries were determined. Plaque size in the aortic root was quantified (C) (**P*<0.05; ***P*<0.01; ****P*<0.001).

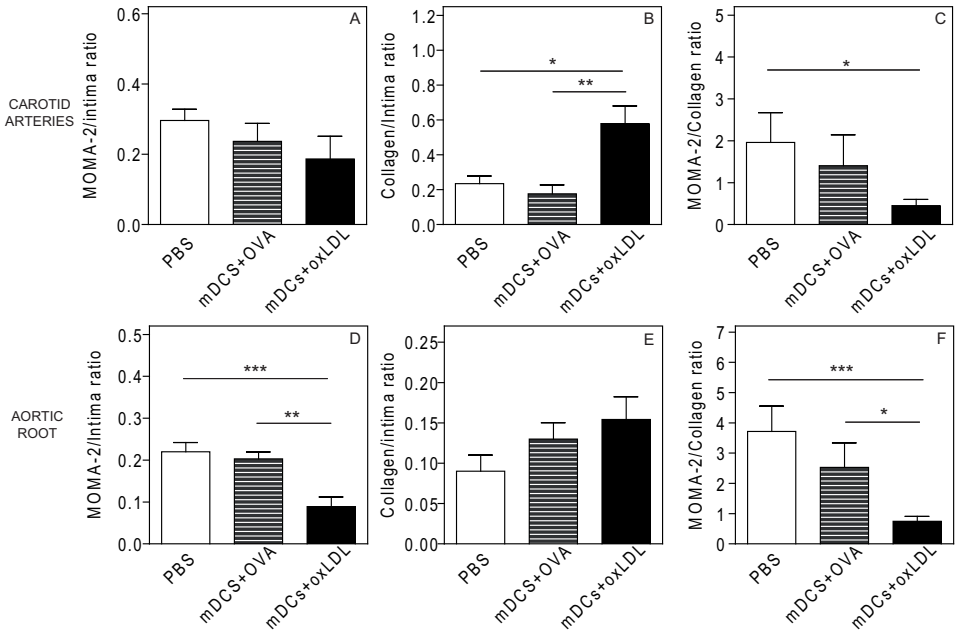

Figure 6: Effect of antigen pulsed dendritic cells on lesion stability
Plaque composition was assessed in the carotid artery (A-C) and in the aortic root (D-F) (**P*<0.05; ***P*<0.01; ****P*<0.001).

Effect on body weight and cholesterol levels

Both body weight and total cholesterol levels were measured at different time points during the experiment. Throughout the experiment, differently treated mice did not vary in body weight (data not shown). Also cholesterol levels were not affected by the DC-treatment. Only at sacrifice, mice treated with oxLDL-pulsed mDCs showed a 27% (P_{anova}=0.0066) lower plasma cholesterol level compared to mice treated with mDCs (PBS: 1538±158 mg/dl; mDCs: 1483±66 mg/dl; mDCs+oxLDL: 1076±101 mg/dl). This reduction did not result from cholesterol lowering in a particular class of lipoproteins (VLDL, LDL or HDL). Nevertheless, we calculated the area under the curve for the cholesterol burden during the entire experiment and again there was no difference between mice treated with mDCs or oxLDL-pulsed mDCs (data not shown). Also, cholesterol levels were not affected by treatment with ovalbumin-pulsed DCs (1492±115 mg/dl).

Induction of an oxLDL-specific humoral response after vaccination

Plasma samples from each mouse were obtained after sacrifice and IgG, IgM and subclass specific antibodies were determined. The T cell-dependent IgG antibodies against oxLDL were increased in mice vaccinated with oxLDL-pulsed DCs (Figure 7A) (P_{anova}<0.0001). Interestingly no significant effect was found on the IgG levels against MDA-LDL which indicates a highly specific response against oxLDL (Figure 7B). No differences in titers of IgM against oxLDL or MDA-LDL were detected (Figure 7C, D).

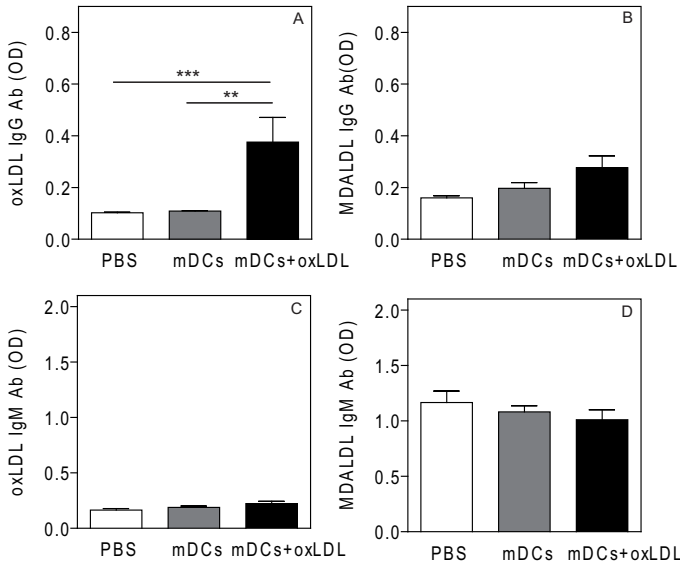

Figure 7: Humoral response against oxLDL and MDA-LDL

IgG and IgM titers were determined in serum in mice treated with PBS (n=9), mDCs (n=11) and oxLDL-pulsed mDCs (n=11) using an ELISA. IgG titers against oxLDL and MDA-LDL are presented in A and B, respectively. Figures C and D show IgM levels against oxLDL and MDA-LDL, respectively (**P<0.01; ***P<0.001).

We also analyzed whether IgG1 or IgG2c mainly contributed to the rise in anti-

oxLDL IgG levels. We observed significantly higher levels of both IgG1 and IgG2c in serum of mice vaccinated with oxLDL pulsed mDCs ($P<0.001$) as determined by ELISA (Figure 8A). Also, IgG1 levels in spleen were elevated ($P<0.01$) as determined by flow cytometry (Figure 8B). There were no differences in the number of IgM producing cells, the number of B cells nor the number of T cells in spleen was affected (data not shown). When we calculated the IgG2c/IgG1 ratio we observed a tendency, especially in the spleen, towards a reduced ratio indicative of a Th2 response ($P=0.08$) (Figure 8C and 8D). We observed that treatment with ovalbumin-pulsed mDCs induced a significant increase in the IgG titers for ovalbumin showing that the ovalbumin-pulsed mDCs effectively induced an immune response against ovalbumin (Figure 9). In addition, the IgG1/IgG2c ratio was not changed, suggesting that the Th1/Th2 ratio was not disturbed in mice treated with ovalbumin-pulsed mDCs.

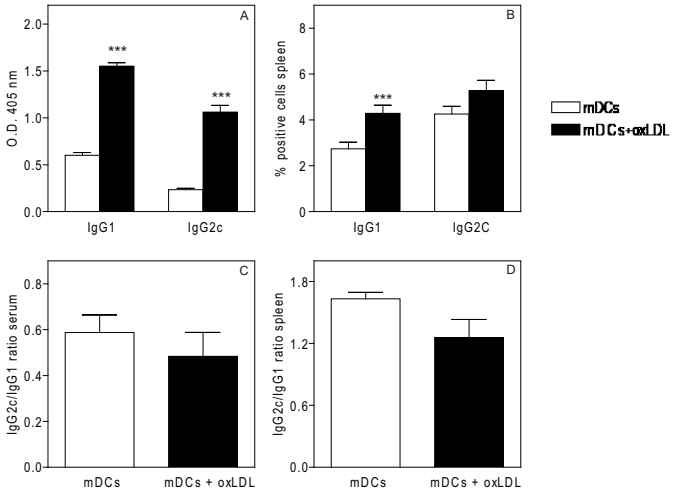

Figure 8: IgG1 and IgG2c response against oxLDL
IgG1 and IgG2c levels in serum (n=10) and spleen (n=5) were determined by ELISA or flow cytometry (A and B, respectively). The ratio of IgG2c to IgG1 in serum and spleen are depicted in C and D, respectively (***$P<0.001$).

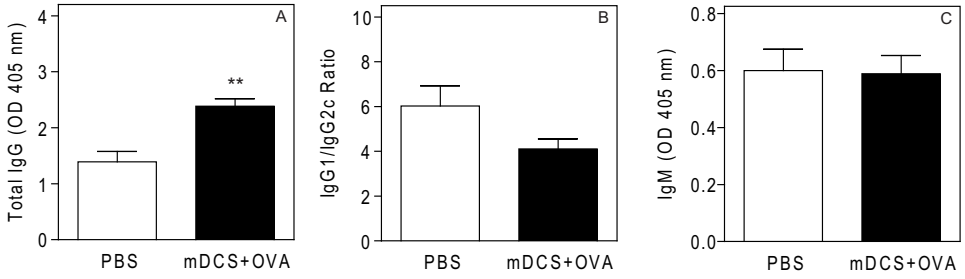

Figure 9: Induction of ovalbumin specific antibodies after injection of ovalbumin-pulsed DCs
Ovalbumin specific Total IgG; IgG1/IgG2c ratio and IgM titers were determined in serum in mice treated with PBS (n=12) mDCs and OVA-pulsed mDCs (n=13) using an ELISA (**$P<0.001$).

Induction of oxLDL-specific T cells in mice treated with oxLDL-pulsed mDCs

We have previously shown that oxLDL induced proliferation of $CD4^+$ T cells and not $CD8^+$ T cells or $CD19^+$ B cells in whole spleen cell cultures after oxLDL treatment (Figure 10).[22] To investigate whether we have induced an oxLDL-specific T cell response, we therefore re-stimulated splenic leukocytes *in vitro* with oxLDL (5 µg/ml) and determined the proliferation of splenic T cells by incorporation with ^{3}H-thymidine. The incubation of oxLDL to splenocytes resulted in a more pronounced T cell proliferation in mice treated with oxLDL-pulsed mDCs (1.8-fold increase, $P<0.01$) and the proliferation was significantly higher than the oxLDL-induced proliferation in the mice treated with mDCs ($P<0.05$) (Figure 11).

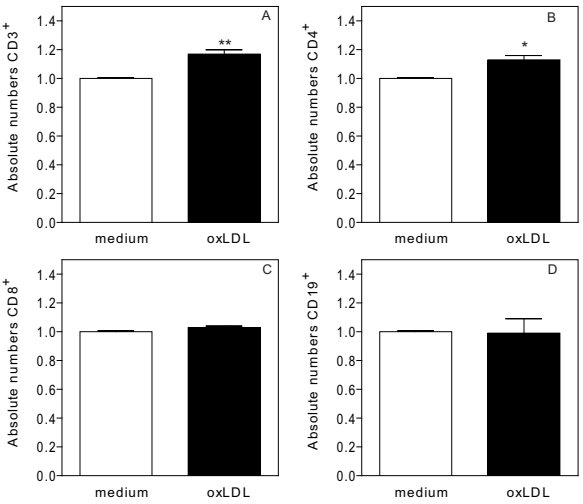

Figure 10: FACS data of spleen cells after oxLDL treatment
Spleen cells were cultured in the presence of oxLDL (5 µg/ml) during 48 hours (n=3) and absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells were determined. Then the stimulation index per spleen was calculated (*$P<0.05$; **$P<0.01$).

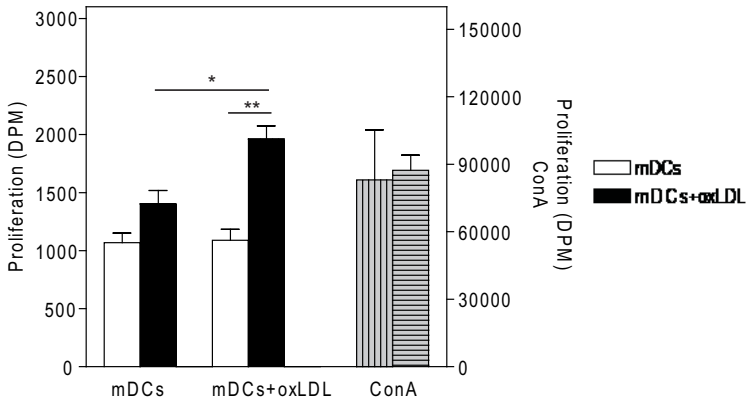

Figure 11: oxLDL-specific proliferation response
Total splenocytes were *in vitro* cultured in the absence (white bars) or presence of oxLDL (black bars). Proliferation was measured by ^{3}H-thymidine incorporation. ConA was used as a positive control (n=5;*P<0.05;**P<0.01).

Cytokine production after *in vitro* stimulation of splenic T cells

We also determined cytokine production by T cells from mice treated with oxLDL-pulsed DCs or unpulsed mDCs 24 hours after stimulation of splenocytes with ConA. We observed a 75% reduction in IFN-γ production by splenic T cells from mice vaccinated with oxLDL-pulsed mDCs compared to T cells from mice treated with mDCs (P=0.001) (Figure 12A). The level of IL-10 and IL-4 production by T cells was equal in both groups (Figure 12B and 12C, respectively).

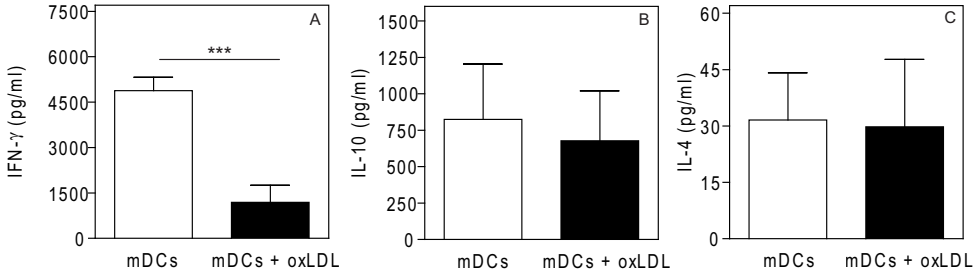

Figure 12: Cytokine production of splenocytes after *in vitro* stimulation
Total splenocytes were *in vitro* cultured with ConA during 24 hours. Cytokine levels were determined in the supernatants (n=5; ***P<0.001).

Cellular response to vaccination strategy

To evaluate the effect of DC treatment on the numbers of different subsets of leukocytes, blood was collected at key time points during the experiment (before injection of DCs, after each injection, before collar placement and at sacrifice) and FACS analysis was performed on leukocytes. We analyzed the numbers of T cells (CD4 and CD8), regulatory T cells ($CD4^+CD25^{high}$), NKT cells ($CD3^+NK1.1^+$). We observed no significant differences in the numbers of blood leukocytes at any time point during the experiment (Table1). We also performed FACS analysis on leukocytes in spleen, liver and mediastinal lymph nodes after sacrifice and again observed no differences between the various groups (data not shown).

Table 1: % of leukocytes in the blood after sacrifice

Markers	PBS	mDCs	mDCs+oxLDL
$CD4^+$	13,32 ± 2,24	12,01 ± 0,37	14,33 ± 1,96
$CD4^+CD25^{high}$	0,87 ± 0,11	1,28 ± 0,08	1,2 ± 0,17
$CD4^+CD62L^{low}$	8,02 ± 2,22	8,52 ± 2,23	8,29 ± 1,36
$CD8^+$	6,73 ± 0,54	6,88 ± 0,61	7,96 ± 0,78
$CD8^+CD62L^{low}$	3,21 ± 0,43	3,55 ± 0,6	3,77 ± 0,13
$CD3^+NK1.1^+$	1,98 ± 0,23	2,78 ± 0,51	2,36 ± 0,12

Inhibition of foam cell formation

Bone-marrow derived macrophages were cultured using L929-conditioned medium (source of M-CSF). The phenotype of the macrophages was tested using flow cytometry and the cultured cells were for more than 90% positive for the macrophage specific markers F4/80 and CD11b, whereas they were less than 10% positive for the DC marker CD11c (Figure 13A). Foam cell formation by the addition of oxLDL was 2.5-fold (P<0.001) lower when the macrophages were incubated with serum from oxLDL-pulsed DC compared to the addition of serum from mice treated with mDCs (Figure 13B), indicating that treatment with oxLDL-pulsed mDCs results in the formation of oxLDL-specific Ab that reduce foam cell formation.

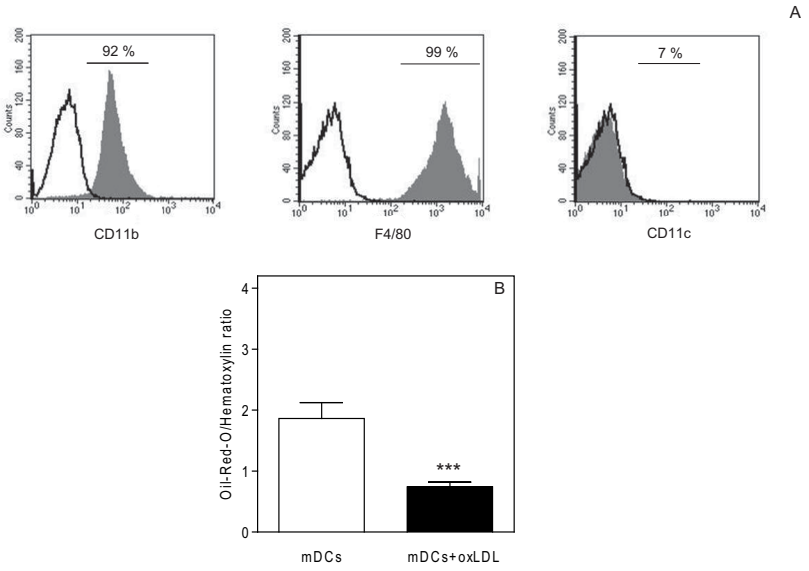

Figure 13: Inhibition of foam cell formation

Bone-marrow derived macrophages were analyzed by flow cytometry. The fluorescence intensity of respectively CD11b, F4/80 and CD11c (grey curves) are depicted in A. The white curves represent the isotype control. B. The amount of Oil-Red-O staining was corrected for the number of cells as indicated by the hematoxylin staining. (pooled sera; n=3), 8 fields per condition (***P<0.001).

Discussion

Oxidation of lipoproteins and oxidative processes play an important role in the initiation and progression of atherosclerosis. In this study we show that vaccination using oxLDL-pulsed DCs effectively reduces atherosclerotic lesion formation in $LDLr^{-/-}$ mice. In healthy vascular tissue, low numbers of DCs reside within the intima, immediately beneath the endothelium and in the adventitia along the external elastic lamina.[23, 24] In atherosclerotic-prone regions of healthy carotid arteries, the DCs accumulate and form clusters at sites subjected to major haemodynamic stress. It has been suggested that the migratory routes of vascular DCs are similar to those in other peripheral tissues. After uptake of antigen, vascular DCs migrate towards regional lymph nodes where they activate T cells. Importantly, plaque progression is linked to a reduced emigration of monocyte-derived DC-like cells from developing lesions.[25] In accordance, histopathological studies on human arterial tissues demonstrate that occasional DCs migrate towards the lymph nodes whereas other DCs stay within the plaques and locally interact with T cells.[26] Also, hyperlipidemia suppresses the migration of skin DCs.[27] Taken together, these studies emphasize the important immunoregulating role of vascular DCs and that the impaired migration of DCs may play an important role in the development of atherosclerosis.

We therefore treated atherosclerosis prone mice before the induction of atherosclerosis with DCs that were loaded *ex vivo* with cupper oxidized LDL. The advantage of using mature pulsed-DCs is that these cells do not need to migrate towards the plaque to engulf oxLDL. The oxLDL-pulsed mDCs used in this study are ready to migrate directly towards the lymphoid tissues where they can interact with lymphocytes and induce oxLDL-specific immune responses.[28] Indeed we showed that CCR7, necessary for migration towards lymphoid organs, is upregulated in the mature DCs used in this study and that injected GFP^{+} DCs homed towards the spleen and lymph nodes. In contrast to pulsing of DCs with oxidized lipids (Bluml *et al.)*[29] we did not observe an effect of oxLDL-priming on LPS-induced DC maturation, which may result from the fact that the amount of added oxLDL (and thus of oxidized lipids) by us was too low to affect the DC maturation. A recent paper by Packard *et al.* shows that foam cell formation as such does not affect the antigen presenting capacity of dendritic cells.[30]

We now show that vaccination using oxLDL-pulsed mDCs reduced atherosclerotic lesion formation in the carotid arteries for 87% after collar placement. Since the initial study of Palinski a number of studies have shown the effectiveness of immunization against oxLDL or apoB-100 peptides in experimental animal models.[31] Immunization leads to a reduction in atherosclerosis ranging from 40 to 70% in mice and rabbits.[9-11, 32] We chose to use oxLDL-loaded DCs for vaccination since it provides us with a very effective method to initiate immune responses to oxLDL. The oxLDL-pulsed DCs present a broad spectrum of epitopes after internalizing and processing the Ag. Therefore DCs are capable of activating a wide range of oxLDL-specific T cells and immune escape is minimized. The use of oxLDL-pulsed mDCs provided us therefore with a very efficient method of manipulating the immune system against multiple epitopes of oxLDL without the requirement of an adjuvant.

The observed reduction in lesion size in the carotid arteries was accompanied by a decrease in macrophage numbers and an increase in collagen, leading to a significantly more stable plaque phenotype**.** Although we only observed a 24% reduction of lesion size in the aortic valve leaflets, the injection of oxLDL-pulsed mDCs did result in more stable atherosclerotic lesions in the aortic root thereby confirming the effect of the treatment on plaque composition in the carotid arteries. The discrepancy in the effect of immunization against oxLDL on plaque size between aortic root and arteries is similar to the observations of Fredrikson *et al.* who showed that immunization against apoB-100 induced a 60% reduction in plaque size in the descending aorta but did not affect the size of the plaques in the aortic root.[10] The differential effect of the treatment using oxLDL-pulsed DCs on atherosclerosis in the carotid arteries and the aortic valves is an interesting, but not entirely new finding. A review paper of Getz *et al.* nicely describes that initiation of atherosclerosis at different sites in the vasculature may involve different molecular pathways.[33] We have however shown that the effect of regulatory T cells on atherosclerosis is similar in the collar-induced atherosclerosis in the carotid arteries and in the aortic root.[22, 34] Therefore it should be noted that the observed difference between the carotid artery and the heart valves is not necessarily the consequence of the collar model. The collar model used in this study is based on the induction of shear stress by the narrowing of the carotid arteries upon placement of the mildly constrictive silicone collars around carotid arteries. Proximal to the collar a decrease in shear stress is observed and the expression of KLF-2 is almost undetectable at this site. Endothelial KLF-2 links local arterial shear stress levels to the expression of vascular tone-regulating genes.[35] The low KLF-2 expression proximal to the collar coincides with the enhanced expression of VCAM-1 and is exactly the site were the atherosclerotic lesions are formed. The collar model is therefore injury-driven but comparable to the human situation where endothelial KLF-2 expression is lowered at the site of atherosclerosis.

During the experiment, cholesterol levels were evaluated. There was no difference in cholesterol burden during the entire experiment but at sacrifice, mice treated with oxLDL-pulsed DCs showed lowered cholesterol levels. This may form an additional interesting long-term effect of the treatment with oxLDL-pulsed DC. However, it cannot explain the enormous decrease in atherosclerotic lesion formation because up to 3 weeks prior to sacrifice, there were no differences in cholesterol levels. *Ex-vivo* generated and antigen-loaded DCs have been used in vaccination protocols in many animal models[36, 37] and have been used to improve immunity in cancer[38] and HIV-infected patients by inducing specific T cells.[39, 40] The attenuation of atherogenesis in our study was accompanied by an increase of T cell-dependent IgG Ab towards oxLDL and not towards MDA-LDL. This indicates that the treatment with oxLDL-pulsed mDCs induced an activation of ox-LDL-specific T cells. Indeed we show that spleen cells from mice treated with oxLDL-pulsed mDCs displayed an increased *in vitro* proliferation towards oxLDL as compared to spleen cells from mDCs-treated mice. In addition, both the enhanced IgG1/IgG2c ratio and the cytokine production of T cells point towards an anti-inflammatory response in the oxLDL-pulsed DCs vaccinated mice as shown by the clear inhibition of the IFN-γ production by ConA stimulated cells in these mice. We and others have previously shown that a reduction in IFN-γ contributes

to a reduction in atherosclerosis.[41] Interestingly, we now observe that the T cell response induced by oxLDL-pulsed DCs shows a reduced Th1 response. By using mDCs loaded with an atherosclerosis-irrelevant antigen, ovalbumin, we showed that the effects observed using oxLDL-pulsed mDCs were due to oxLDL-specific responses and not to side-effects inflicted by the use of antigen-pulsed mDCs. Indeed, although ovalbumin-specific IgG antibodies were induced, lesion size and composition were not changed in mice treated with ovalbumin-pulsed mDCs, nor were cholesterol levels affected.

In a previous study we showed that oral tolerance against oxLDL in $LDLr^{-/-}$ mice results in the induction of TGF-β producing regulatory $CD4^{+}CD25^{+}FoxP3^{+}$ T cells that inhibit lesion formation.[22] Oral tolerance to HSP60 also reduces atherosclerosis by inducing HSP60 specific regulatory T cells that produce TGF-beta and IL-10. The induction of oral tolerance to oxLDL and HSP60 was not accompanied by the induction of IgG or IgM specific for the oraly administered antigen.[34] These findings are in contrast to the present study where the treatment with oxLDL-pulsed mDCs induced anti-oxLDL antibodies, a reduction in IFN-gamma production by oxLDL-specific T cells and we did not observe an effect on the number of Tregs. We can conclude from these studies that both oral tolerance towards oxLDL as inducing a oxLDL-specific immune response can be beneficial in the treatment of atherosclerosis.

We also showed that serum of mice treated with oxLDL-pulsed mDCs reduced the formation of foam cells. These mice had higher titers of oxLDL-specific IgG Ab and we argue that the oxLDL-specific IgG can inhibit foam cell formation via complex formation with oxLDL. This is in agreement with the findings of the group of Witztum who showed that monoclonal IgG Fab Ab directed to oxLDL blocked foam cell formation in macrophages.[42] Also, Caligiuri *et al.* showed that sera from $ApoE^{-/-}$ mice, immunized with phosphorylcholine reduced the uptake of oxLDL by macrophages.[43] In addition, both Zhou *et al.* and Freigang *et al.* demonstrated an inverse correlation between lesion size and anti-MDA-LDL IgG levels in mice immunized with MDA-LDL.[11, 44] These data suggest that the presence of oxLDL-immune complexes play an atheroprotective role and it is suggested that the protective effect may be partially mediated via the inhibitory Fc receptor IIB.[45]

Taken together, this strategy of DC vaccination is a new powerful vaccination method which provides us with a highly efficient route to modulate the immune responses to oxLDL by inducing oxLDL-specific T cells with a reduced Th1 response and enhanced levels of anti-oxLDL IgG. Therefore, the approach of DC mediated vaccination against oxLDL is possibly applicable in young adults to inhibit the initiation of atherosclerosis. Additional studies on the effect of DC therapy on progression of atherosclerosis to eventually implement the therapy in patients need to be performed, but studies using anti-apoB100 antibodies indicate that these antibodies may induce inhibition of plaque progression or may even induce plaque regression, indicating that an effect of DC therapy on pre-existing lesions may be relevant.[32, 46]

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