Regulation of T cell responses in atherosclerosis

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Citation

Puijvelde, G. H. M. van. (2007, June 28). *Regulation of T cell responses in atherosclerosis*. Retrieved from https://hdl.handle.net/1887/12149

Version:	Corrected Publisher's Version
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Chapter 4

Immunotherapy of atherosclerosis using dendritic cells

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-Submitted for publication-

Abstract
Modification of lipoproteins plays an important role in the development of atherosclerosis. 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 DCs to LDLr$^{-/-}$ mice and examined the effects on atherosclerosis. Bone marrow derived DCs were cultured for 10 days in the presence of GM-CSF. Immature DCs were matured by LPS and pulsed with cupper oxidized LDL (Cu-oxLDL). These cells were transferred to LDLr$^{-/-}$ mice every other day before induction of atherosclerosis by Western-type diet feeding. Transfer of oxLDL-pulsed DCs resulted in a 92% and 87% reduction in carotid artery lesion size compared to the PBS-treated mice or mice treated with mature DCs (mDCs), respectively ($P<0.001$). Lesion size in the aortic valves was not affected but we observed at both sites an increase in plaque stability. The reduction in atherosclerosis was accompanied by a 3.8-fold increase ($P<0.05$) in Cu-oxLDL specific IgG levels whereas the levels of MDA-LDL-specific IgG and IgM were not significantly affected. In addition, we also showed that the serum of mice treated with oxLDL-pulsed DCs reduced the formation of foam cells as compared to serum from PBS-treated mice or mice treated with mDCs. We conclude that oxLDL-pulsed DCs induce an enhanced production of anti-oxLDL IgG levels which leads to a reduction in atherosclerosis by modulating the immunostimulatory effects of oxLDL. These data indicate that vaccination with oxLDL-pulsed DCs provides a novel and powerful strategy in the treatment 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. Also, peroxidization of polyunsaturated fatty acids in phospholipids and cholesteryl esters generates highly reactive breakdown compounds such as malondialdehyde (MDA) and 4-hydroxynonenal that cause cell damage and local inflammation.[9-11] 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.[12-14]

OxLDL also plays a role in the maturation of dendritic cells (DCs). DCs are the most potent antigen-presenting cells of the immune system.[15-17] In the periphery they act as sentinels for the innate immune system whereas in the lymph nodes they are the key messengers in adaptive immunity. 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.[18,19]

Due to their potent capacity to stimulate T cells, DCs are being investigated in vaccine and therapy approaches.[20,21] We wanted to assess the use of oxLDL-pulsed DCs as an immunotherapy for atherosclerosis. DCs obtained from the bone-marrow can be pulsed *ex vivo* by inducing maturation in the presence of oxLDL. Such cultivation of DCs will mimic the *in vivo* processing of oxLDL by DCs. In this study we show that repeatedly injection of oxLDL-pulsed DCs induced an enormous reduction in lesion size and increased the production of oxLDL-specific antibodies (Ab). The high titers inhibit foam cell formation and thus prevent the negative effects of oxLDL on the arterial wall. In conclusion, these data indicate that vaccination with oxLDL-pulsed DCs provides a novel and powerful strategy in the battle against atherosclerosis.

Methods

Animals

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*.

Media and reagents

The cell culture medium used for dendritic cells was IMDM (Cambrex, Belgium) supplemented with 8 % FBS (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.[22] The isolated LDL was dialyzed against phosphate buffered saline (PBS) with 10 M 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.[23]

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 cupper-oxidized LDL. 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 1.5·10^6 DCs (oxLDL-pulsed or unpulsed mDCs) in 200 μl PBS (n = 11). DCs from the UBC-GFP mice were identically isolated en cultured.

Cytospin

300 μl of cell suspensions 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.

Induction of atherosclerosis

Male LDLr$^{-/-}$ mice were injected 3 times intravenously (1 injection every other day) with $1.5 \cdot 10^6$ 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.[24] During the experiment, plasma samples were obtained by tail vein bleeding. 7 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 5 μm 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 and Oil-Red-O stained sections of heart valves were used for morphometric analysis of atherosclerotic lesions as described previously.[24] 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 site of maximal stenosis was used for assessment. The images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK).

Flow Cytometry (FACS analysis)

Antibodies used for staining were purchased from eBiosience, Belgium. Staining was done in PBS in the presence of 1 % mouse serum. FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest 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).

ELISA for IgM and IgG antibodies against LDL, MDA-LDL and Cu-oxLDL

Antibodies against LDL, MDA-LDL and Cu-oxLDL were determined according to Damoiseaux et al.[25] 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 Ab

IgG1 and IgG2A levels were detected using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Fransisco, USA) according to manufacturers' instructions. Briefly, high binding plates were coated overnight with 50 μl Cu-oxLDL (5 μg/ml). Serum samples were 1:1 diluted in PBS. IgG1 and IgG2A levels were detected using a spectrophotometer at 405 nm.

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 $\cdot 10^6$ cells/well) in cRPMI $^+$ 10% FCS. After 4 hours of incubation, cells were washed and resuspended in cRPMI without FCS. After 4 hours, a mixture of oxLDL (10 μg/ml) plus an equal volume of mouse serum (2 fold diluted, serum 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 analysed using the Leica DM-RE microscope and LeicaQwin software.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Differences between different treated groups were assessed with parametric or non-parametric ANOVA followed by a t-test using the Instat3 software. To measure the effects on cholesterol, the repeated measures ANOVA was used. Probability values of $P < 0.05$ were considered significant.

Results

Effect of oxLDL on dendritic cells

Firstly we investigated whether DCs did engulf oxLDL and whether the uptake of lipoproteins by DCs did affect the maturation of DCs. During maturation, DCs

upregulate the expression of costimulatory molecules and the maturation level of DCs can thus be followed by flow cytometry of several typical markers (CD40, CD80, CD86, MHCII, and CD1d). 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 and found that increasing oxLDL concentrations induced an increasing lipid accumulation. However, oxLDL levels exceeding 7.5 μg/ml induced a concentration dependant increase in cell death and a lowering of the expression of the DC-specific markers (data not shown). Therefore we have chosen a concentration of 7.5 μg/ml which induced optimal lipid loading but no cell death.

We treated immature DCs (Figure 4.1A) with the TLR4 agonist, LPS (1 μg/ml), in the absence or presence of oxLDL (7.5 μg/ml). After 24 hours of incubation, DCs were collected and stained with Oil-Red-O and analyzed for surface markers by flow cytometry. The addition of oxLDL to both imDCs and mDCs resulted in lipid accumulation in more than 95% of the DCs (Figure 4.1B and 4.1C respectively). Treatment with LPS resulted in a typical mature DC cell surface phenotype showing high expression of CD11c (a specific marker for mouse DCs), MHCII, 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 4.1D).
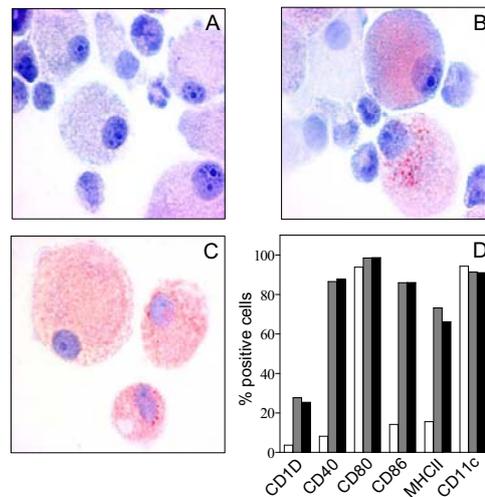

Figure 4.1: The effect of oxLDL on dendritic cells. Immature DCs were treated with LPS (1 μg/ml) in the absence or presence of oxLDL (7.5 μg/ml). After 24 hours, DCs were collected. Oil-Red-O staining of DCs: immature DCs in the absence of oxLDL (A), in the presence of oxLDL (B) and mature DCs in the presence of oxLDL (C). Expression levels of surface markers analysed by FACS on immature DCS (white bars), mature DCs (grey bars) and mature oxLDL-pulsed DCs (black bars).

Homing of dendritic cells.

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 $1.5 \cdot 10^6$ DCs into LDLr$^{-/-}$ mice. We injected both mature unpulsed and mature oxLDL-pulsed

GFP-DCs and 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 blood, DCs did migrate towards the lung and liver, and towards organs involved in immune responses such as spleen, mediastinal and inguinal lymph nodes (Figure 4.2). Mature unpulsed and mature oxLDL-pulsed DCs displayed an identical distribution pattern.

Effect of vaccination using oxLDL-pulsed DCs on atherosclerosis

To evaluate the effect of vaccination using oxLDL-pulsed DCs on de novo atherosclerosis, LDLr$^{-/-}$ were injected intravenously 3 times (day -8, -6 and -3) with either PBS (n = 8), mDCs (n = 11) or oxLDL-pulsed DCs (n = 11). 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 and subsequent Western-type diet feeding. Seven weeks after collar placement, mice were sacrificed and plaque formation in the right carotid artery and the aortic root were analyzed using the hematoxylin/eosin staining and hematoxylin/Oil-Red-O staining, respectively. Figure 4.3A-C show representative examples of hematoxylin/eosin staining in the carotid arteries. Injection of oxLDL-pulsed DCs resulted in a significant 92% (48578±9231 μm^2 vs

Figure 4.2: Homing of GFP$^+$-DCs. 1.5·10^6 mature GFP$^+$-DCs (grey 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. GFP$^+$ cells as percentage of the total number of leukocytes in the different organs are shown.

4024±504 μm^2; $P<0.001$) or 87% (31919.8±7914 μm^2 vs 4024±504 μm^2; $P<0.001$) reduction in plaque size in the carotid arteries compared to the PBS-treated or mDC-treated groups, respectively (Figure 4.3D). Also a concomitant reduction in intima/lumen ratio of 87% (0.560±0.097 vs 0.071±0.009; $P<0.001$) or 85% (0.461±0.089 vs 0.071±0.009; $P<0.001$) was observed (Figure 4.3E). In contrast, we did not observe significant effects of treatment with dendritic cells on plaque size in the aortic roots (PBS: 199411±17408 μm^2; mDCs: 293181±36193 μm^2; mDCs plus oxLDL: 224983±48546 μm^2) (Figure 4.3F).

Plaque morphology was assessed using the MOMA-2 staining for detecting macrophages and the Masson's Trichrome method was used for visualizing collagen fibers. In the carotid arteries we observed non-significant decreases in the MOMA-2/intima ratio (Figure 4.4A). The macrophage content was also reduced in the aortic root, where we saw a significant 2.5-fold reduction compared to the PBS-treated group ($P<0.05$) and a non-significant 1.9-fold reduction in MOMA-2/intima ration compared to the mDC-treated group (Figure 4.1D). The collagen content of the intima was increased in both the carotid arteries (Figure 4.4B; $P<0.05$) and the aortic root (Figure 4.4E). Additionally, we calculated the MOMA-2/collagen ratio as an indicator for plaque stability.
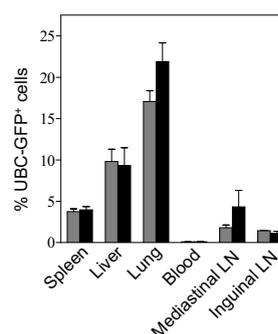
There was a significant 4.3-fold ($P<0.05$) and 4.1-fold ($P<0.05$) reduction in MOMA-2/collagen ratio in the group treated with oxLDL-pulsed DCs compared to the group treated with PBS and a significant 3.9-fold and 2.8-fold reduction compared to the group treated with mDCs in both the carotid arteries and aortic root, respectively (Figure 4.4C and 4.4F). This reduced MOMA-2/collagen ratio indicated a more stable plaque phenotype in the mice treated with oxLDL-pulsed DCs.
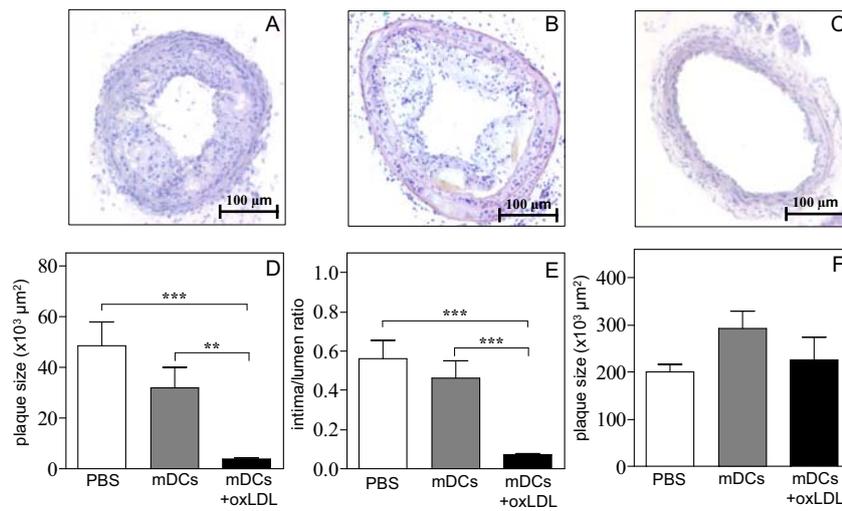

Figure 4.3: Effect of DC vaccination on atherosclerosis. Representative sections of lesions in the carotid arteries of PBS-treated (A), mDC-treated (B) and oxLDL-pulsed DC-treated (C) mice. Lesion size (D) and intima/lumen ratio (E) in the carotid arteries were determined. Plaque size in the aortic root was quantified after hematoxylin/Oil-Red-O staining (F). (n=7-11 per group; $**P<0.01$, $***P<0.001$)

Effect of vaccination on total cholesterol levels

Both body weight and total cholesterol levels were measured at different time points during the experiment. There were no significant differences in body weight between the different groups throughout the entire experiment. As shown in figure 4.5A, cholesterol levels of the PBS and mDC-treated groups kept on increasing in time, whereas the cholesterol levels in the group treated with oxLDL-pulsed DCs levelled off after 7 weeks. The repeated measurements ANOVA did not result in significant differences over time. It remains however interesting that at sacrifice, mice treated with oxLDL pulsed DCs showed significant 30% ($P<0.05$) and 27% ($P<0.01$) lower cholesterol levels compared to mice treated with PBS and mDCs, respectively. The reduction did not result from the lowering of one particular class of lipoproteins (VLDL, LDL or HDL) as depicted in the lipoprotein profile (Figure 4.5B).

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 Cu-oxLDL were significantly higher in mice vaccinated with
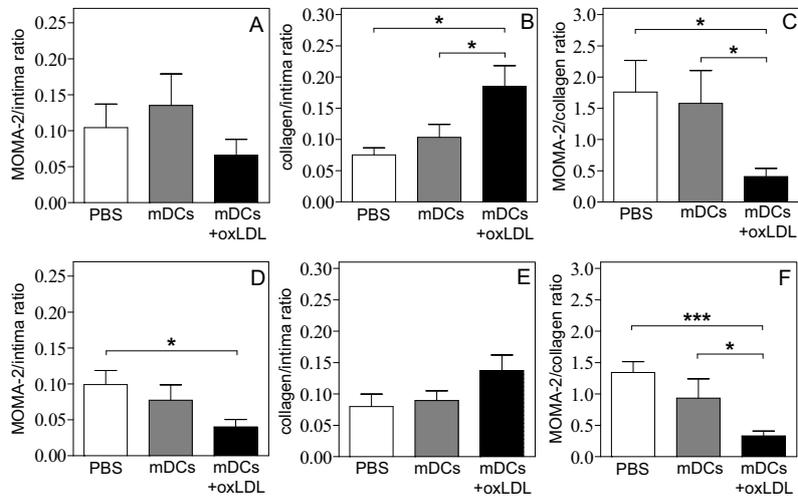
Figure 4.4: Plaque morphology. LDLr$^{-/-}$ mice were treated with PBS, mDCs or mature oxLDL-pulsed DCs. Subsequently mice were put on a Western-type diet and 10 weeks later plaque composition was assessed in the carotid artery (A-C) and in the aortic root (D-F). The amount of macrophages was determined as the MOMA-2 positive area per intima area (A and D), the amount of collagen was determined by quantifying the blue collagen staining of the Masson's Trichrome stain within the plaque (B and E). The plaque stability was quantified as MOMA-2/collagen ratio (C and F) (n=7-10 per group; $^{*}P<0.05$), $^{***}P<0.001$
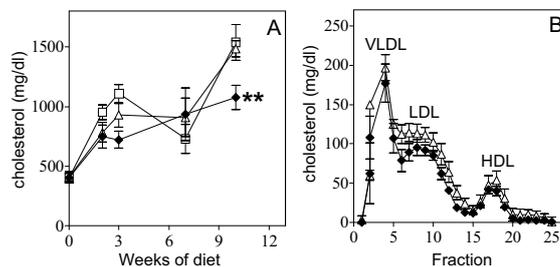

Figure 4.5: Effect on total plasma cholesterol. Cholesterol levels were determined in plasma of the different mouse groups (PBS (open squares), mDCs (open triangles) and oxLDL-pulsed mDCs (closed diamond)) at the indicated time points (A). Serum was separated on a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles (B). (n=8-11 per group; $^{**}P<0.01$).

oxLDL-pulsed DCs compared to the mice treated with PBS or mDCs (Figure 4.6A) ($P< 0.001$ and $P< 0.01$, respectively). Interestingly no significant effect was found on the IgG levels against MDA-LDL which indicates a highly specific response against Cu-oxLDL (Figure 4.6B). No differences in titers of IgM against MDA-LDL or Cu-oxLDL were detected between the various groups (Figure 4.6C and D, respectively). We also analyzed whether IgG1 or IgG2a mainly contributed to the rise in anti-oxLDL IgG levels and we observed significantly higher levels of both IgG1 and IgG2A in the mice treated with oxLDL-pulsed DCs compared to the mDC-treated group indicating that the production of anti-oxLDL antibodies was not restricted to a Th1 or Th2 response (data not shown).
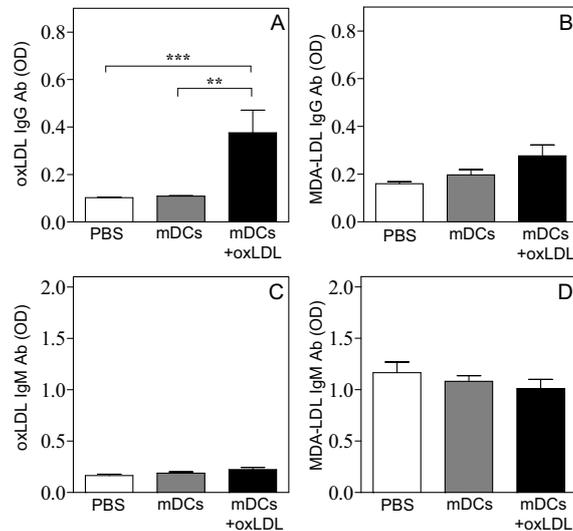
Figure 4.6: Humoral response against oxLDL and MDA-LDL. At the end of the experiment, IgG and IgM titers were determined in serum of mice treated with PBS, mDCs and mature oxLDL-pulsed DCs using an ELISA. IgG titers against oxLDL and MDA-LDL are presented in A and B, respectively. Figures C and D show IgM levels against oxLDL and MDA-LDL, respectively. (n=8-11 per group; $^*P<0.05$).

Cellular response to vaccination strategy

To evaluate the effect of DC treatment on a number of different subsets of leukocytes, blood was collected before injection of DCs, after each injection, before collar placement and at sacrifice and FACS analysis was performed on leukocytes. We observed no significant differences in number of blood $CD8^+$ or $CD4^+CD25^+$ cells at any time point during the experiment. We also performed FACS analysis on spleen, liver and mediastinal lymph nodes after sacrifice. There were no differences in number of T cells, regulatory T cells, $CD11c^+$ or $CD1d^+$ cells (data not shown).

Inhibition of foam cell formation

Macrophages were obtained by culturing bone marrow derived cells from C57Bl6 mice in L929-conditioned medium (source of M-CSF). The phenotype of the macrophages was tested using FACS analysis. Cells were >90% positive for F4/80 and CD11b and <10% positive for CD11c and thus considered to be macrophages (Figure 4.7A-C). Foam cell induction in these macrophages by incubation with oxLDL was hampered by serum from animals treated with oxLDL-pulsed DC (Figure 4.7D). Lipid loading in the foam cells was 3.8 ($P<0.01$) and 2.5 ($P<0.001$) times lower using serum from mice treated with oxLDL-pulsed DC as compared to serum from PBS or mDC-treated mice, respectively.
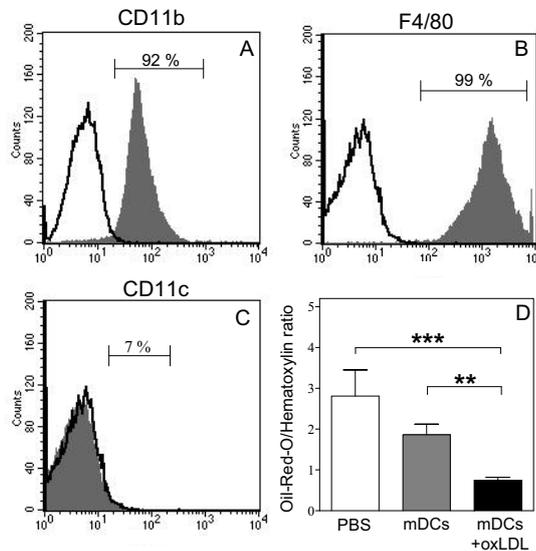
Figure 4.7: Inhibition of foam cell formation. Bone-marrow derived macrophages were analysed by FACS analysis. The fluorescence intensity of respectively CD11b, F4/80 and CD11c (grey curves) are depicted in A, B and C, respectively. The white curves represent the isotype control. The amount of Oil-Red-O staining of macrophages was corrected for the number of cells as indicated by the hematoxylin staining. The addition of mouse serum from mice treated with oxLDL-pulsed DCs (black bars) inhibits the uptake of oxLDL by macrophages as compared to the serum from mice treated with PBS (white bars) and mDCs (grey bars) (D). (n=3, 8 fields per condition) $**P<0.01$; $***P<0.001$.

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 is a successful strategy in reducing arterial atherosclerotic lesion formation in LDLr$^{-/-}$ mice. In healthy vascular tissue low numbers of DC reside within the intima, immediately beneath the endothelium and in the adventitia along the external elastic lamina.[26,27] In atherosclerosis-prone regions of healthy carotid arteries, the DCs accumulate and form clusters at sites subjected to major haemodynamic stress. This accumulation of DCs at sites prone to develop atherosclerosis has already been established in children from 8 weeks to 10 years old.[26,27] So, vascular DCs become already activated in very early stages of atherosclerosis and induce a primary immune response against the antigens present in the adventitia such as oxLDL. In the advanced atherosclerotic plaques the number of DCs increases by invasion of DCs from the adventitia and from blood. After antigen uptake within the lesions, activated DCs form clusters with T and NKT cells in the shoulder regions of the plaques and may thus play an important role in plaque destabilization.[28,29] 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 tend to migrate towards regional lymph nodes where they activate T cells. Importantly, plaque progression was linked to a reduced emigration of monocyte-derived DC-like

cells from developing lesions.[30] In accordance, histopathological studies on human arterial tissues demonstrated that some DCs migrate towards the lymph nodes while other DCs stay within the intima and interact with T cells.[31] Also, hyperlipidemia suppressed the migration of skin DCs.[32] Taken together, these studies emphasize the important immunoregulatory 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 with DCs that were loaded *ex-vivo* with modified LDL to prime the immune response against modified LDL, which is one of the main pro-inflammatory antigens in atherosclerosis. At present we show that the use of oxLDL-pulsed DCs resulted in a 92% or 87% reduction in lesion seize in the carotid arteries compared to the PBS or the mDC-treated control groups, respectively. *Ex-vivo* generated and antigen-loaded DCs have been used in vaccination protocols in many animal models[33,34] and have been used to improve immunity in cancer[35] and HIV-infected patients.[36,37] Since the initial study of Palinski[38], a number of studies have shown the effectiveness of immunization with oxLDL or apoB100 peptides as an immunotherapy for atherosclerosis in experimental animal models leading to a reduction in atherosclerosis ranging from 40 to 70% in mice and rabbits.[12–14,39] The use of oxLDL loaded dendritic cells provided us with a simple but very sufficient method of manipulating the immune system and DC-therapy does not require the use of adjuvants. Also, DCs present a broad spectrum of epitopes after internalizing and processing the Ag. Therefore DCs are capable of activating a wide range of Ag-specific T cells and immune escape is minimized. Additionally, we showed that oxLDL is easily engulfed by immature DCs and that the presence of oxLDL during maturation also resulted in the uptake of lipids as shown by the Oil-Red-O staining. By injecting GFP$^+$ DCs we showed that after 3 days both mature unpulsed and mature oxLDL-pulsed DCs homed in an identical manner to various organs including lymph nodes and spleen, where they can interact with other immune cells and thus induce immune responses, as observed by others.[40]

The observed reduction in lesion size in the carotid arteries was accompanied by a decrease in macrophage numbers and an increase in collagen fibers, leading to a significantly more stable phenotype in the oxLDL-pulsed DC group as compared to both the PBS-treated and mDC-treated group. In contrast, we did not observe a significant reduction in lesion size in the aortic valve leaflets. This discrepancy is similar to that observed by Fredrikson et al. who showed that immunization with apoB-100 induced a 60% reduction in plaque size in the descending aorta but did not affect the size of the subvalvular plaques.[13] On the other hand, we did observe a significant reduction in the number of macrophages in the aortic sinus, indicating a reduced inflammatory status of the lesions in the valve area and we did observe an increase in collagen fibers. Taken together, these data indicated a more stable phenotype in the aortic root and confirmed the effect of oxLDL-pulsed DCs on the composition of the plaque in the carotid arteries.

During the experiment, cholesterol levels were evaluated. Interestingly, after DC vaccination and 10 weeks of diet, a significant reduction in total cholesterol levels in mice treated with oxLDL-pulsed DCs was observed. The lowering of total cholesterol observed was not due to a change in the lipoprotein profile as

shown by SMART analysis. Although this decrease of cholesterol cannot explain the enormous decrease in atherosclerotic lesion formation, since the overall cholesterol load during the experiment was comparable between the two groups, it may form an additional interesting long-term effect of treatment with oxLDL pulsed DCs. The reduction in cholesterol levels observed is in accordance with Freigang et al. who showed a significant reduction in total plasma cholesterol in MDA-LDL immunized LDLr$^{-/-}$ mice after 15 weeks of diet.[14]

The lowering of cholesterol was accompanied by an increase of oxLDL-specific IgG antibodies. We did not detect differences in IgM levels when comparing the three groups. However, we did observe an increase in T cell-dependent IgG Ab towards Cu-oxLDL and not towards MDA-LDL in the group treated with oxLDL-pulsed DCs. This indicates that the treatment with oxLDL-pulsed DCs induced an activation of Cu-oxLDL-specific T cells. In addition, we also showed that the serum of mice treated with oxLDL-pulsed DCs reduced the formation of foam cells as compared to serum from PBS or mDC-treated mice. These mice have 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.[41] Also, Caligiuri et al. showed that serum from ApoE$^{-/-}$ mice, immunized with phosphorylcholine reduced the uptake of oxLDL by macrophages. 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.[14,42] These data suggest that the presence of oxLDL-immune complexes play an atheroprotective role.

Taken together, this strategy of DC vaccination provides a highly efficient route to modulate the immune responses to oxLDL during atherosclerosis and is a new efficient vaccination strategy that is practically feasible.

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