Vaccination using oxidized low-density lipoprotein-pulsed dendritic cells reduces atherosclerosis in LDL receptor-deficient mice

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Aims
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 LDL receptor-null (LDLr

Methods and results
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 mDCs were transferred three times to LDLr

Conclusion
These data indicate that vaccination with oxLDL-pulsed mDCs provides a novel and powerful strategy for the immunomodulation of atherosclerosis.

Keywords
Atherosclerosis • Immunotherapy • Dendritic cells • Antigen-presenting cells

1. 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 have 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.

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system. Immature DCs (iDCs) 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 up-regulate the expression of co-stimulatory 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 co-stimulatory molecules and the increased ability to stimulate T cells.

Due to their potent capacity to stimulate T cells, DCs are being investigated in vaccine and therapy approaches. 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.

2. Methods

2.1 Media and reagents

Cell culture medium for the DCs was IMDM (Cambrex, Belgium) supplemented with 8% FCS (PAA, Germany), 100 U/mL streptomycin/penicillin (PAA), 2 mM glutamax (Invitrogen, The Netherlands), and 20 µM β-mercaptopethanol. LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave et al.19 The isolated LDL was dialysed against phosphate-buffered saline (PBS) with 10 µM EDTA (pH 7.4) for 24 h at 4°C and oxidized by exposure to 10 µM CuSO4 at 37°C for 20 h as described previously.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) (cRPMI). For the culturing of bone marrow-derived macrophages, cRPMI with 20% FCS was used.

2.2 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 LDL receptor-null (LDLr–/–) mice and UBC-GFP mice were obtained 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.

2.3 Generation and injection of bone marrow-derived DCs

For each injection time point, bone marrow cells were isolated from the tibia and femora of three C57Bl/6j mice. Cells were immediately pooled and cultured during 10 days in complete IMDM in the presence of granulocyte-macrophage colony-stimulating factor. After 10 days of culture, iDCs were activated with 1 µg/mL of lipopolysaccharide (LPS from Salmonella typhosa, Sigma Aldrich, Zwijndrecht, The Netherlands) during 24 h. 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.

2.4 Assessment of atherosclerosis

Male LDLr–/– mice were injected three times intravenously (one injection every other day) with 1.5 × 106 DCs or saline prior to Western-type diet feeding. Seven weeks after collar placement, mice were sacrificed and tissues were harvested after in situ perfusion using PBS and subsequent perfusion using formalin. Fixed tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen, and stored at −20°C until further use.

2.5 Histological analysis and morphometry

Transverse cryosections were prepared in a proximal direction from the carotid bifurcation. Cryosections were stained with haematoxylin (Sigma Aldrich) and eosin (Merck Diagnostica, Germany) or with the Masson's trichrome method (Sigma Aldrich). 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 × 106 DCs (oxLDL-pulsed or unpulsed mDCs) in 200 µL of PBS (n = 11). DCs from the UBC-GFP mice were identically isolated and cultured.

2.6 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% NH₄Cl in 0.01 M Tris/HCL, pH 7.2. Splenocytes were cultured in triplicates at 2 × 10⁶ cells/well in the presence or absence of oxLDL (5 μg/mL) in complete RPMI 1640 (cRPMI). Concanaavalin A (ConA, Sigma-Diagnostics, MO, USA) (2 μg/mL) was used as a positive control. After 24 h, 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 h with 1 μCi/well of ³H-thymidine (Amersham Biosciences, The Netherlands) and the amount of ³H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R) as the number of disintegrations per minute.

2.7 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 × 10⁶ cells/well) in cRPMI. Cells were starved for 4 h after which a mixture of oxLDL (10 μg/mL) + an equal volume of mouse serum (two-fold diluted sera from two mice were pooled) was added. This mixture of oxLDL and mouse serum was made 30 min 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 haematoxylin. The amount of Oil-Red-O staining was corrected for the number of cells as indicated by the haematoxylin staining. Eight random fields per condition were analysed using the Leica DM-RE microscope and LeicaQwin software.

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

2.9 Online materials and methods
For more details regarding the cholesterol assay, cytopsin, flow cytometry, and the used ELISAs, see Supplementary material online.

3. Results

3.1 Effect of oxLDL on DCs
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 imDCs. After 24 h of incubation, we stained the intracellular lipid accumulation in the DCs with Oil-Red-O and analysed 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 >95% of the DCs (see Supplementary material online, Figure S1B and C) compared with the immature control incubated DCs (see Supplementary material online, Figure S1A).

During maturation, DCs up-regulate the expression of co-stimulatory molecules and the maturation level of DCs can thus be followed by flow cytometry for several specific markers (CD40, CD80, CD86, MHCI, CD1d, and CCR7). We treated imDCs 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 mDC surface phenotype showing high expression of CD11c (a specific marker for mouse DCs), CCR7 (necessary for migration towards lymphoid organs), MHCI, CD1d, and the co-stimulatory molecules CD40, CD80, and 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 (see Supplementary material online, Figure S1D).

3.2 DCs home towards lymphoid organs after iv 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 × 10⁶ DCs into LDLr⁻/⁻ mice. We injected both mature unpulsed and mature oxLDL-pulsed GFP-DCs. Seventy-two hours after injection, mice were sacrificed and GFP expression was determined in different tissues using flow cytometry. Although 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, and mediastinal and inguinal lymph nodes (see Supplementary material online, Figure S2). Mature unpulsed and mature oxLDL-pulsed DCs displayed an identical distribution pattern.

3.3 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 three 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 and Western-type diet was continued for 7 weeks whereafter mice were sacrificed. Plaque formation in the carotid artery and the aortic root was analysed using the haematoxylin/eosin staining and haematoxylin/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 1A–C shows representative examples of haematoxylin/eosin staining in the carotid arteries. Injection of oxLDL-pulsed mDCs resulted in an 87% reduction in plaque size in the carotid arteries (PBS, 48.578 ± 9.231 μm²; mDCs, 31.919.8 ± 7914.3 μm²; mDCs + oxLDL, 4023.5 ± 504.1 μm²; Pa nova = 0.0009) (Figure 1D). 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; Pa nova = 0.0003) was observed (Figure 1E). In the aortic root, the treatment with
oxLDL-pulsed DCs induced a non-significant 24% decrease in plaque size (PBS, 228.667 ± 32.747 μm²; mDCs, 293.181 ± 36.193 μm²; mDCs + oxLDL, 224.983 ± 48.546 μm²) (Figure 1f). Plaque morphology was assessed by the number of macrophages and the presence of collagen fibres. 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 2A) and the aortic root (Figure 2D), whereas the collagen content of the intima was increased in both the carotid arteries (P<0.0145) (Figure 2B) and the aortic root (Figure 2E). In addition, we calculated the MOMA-2/collagen ratio as an indicator for plaque stability. Significant 3.9- and 2.8-fold reductions were observed in both the carotid arteries (P<0.0097) and the aortic root, respectively (P<0.00097) (Figure 2C and F). 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. 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 (see Supplementary material online, Figure S3), whereas it also did not affect plaque composition at these sites (see Supplementary material online, Figure S4). In contrast, oxLDL-pulsed DCs did lower the plaque size in the carotid artery and did beneficially lower the macrophage/collagen ratio in the lesions in both the carotid artery and the aortic root.

3.4 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<0.0066) lower plasma cholesterol level compared with those 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, when we calculated the area under the curve for the cholesterol burden during the entire experiment, 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).

3.5 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 Cuox-LDL were increased in mice vaccinated with oxLDL-pulsed DCs (Figure 3A) (P<0.0001). Interestingly, no significant effect was found on the IgG levels against MDA-LDL which indicates a highly specific response against Cuox-LDL (Figure 3B). No differences in titres of IgM against Cuox-LDL or MDA-LDL were detected (Figure 3C and D). We also analysed 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 (see Supplementary material online, Figure S5A). Also, IgG1 levels in spleen were elevated \( (P < 0.01) \) as determined by flow cytometry (see Supplementary material online, Figure S5B). 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 \( (P = 0.08) \) (see Supplementary material online, Figure S5C and D).

We observed that treatment with ovalbumin-pulsed mDCs induced a significant increase in the IgG titres for ovalbumin showing that the ovalbumin-pulsed mDCs effectively induced an immune response against ovalbumin (see Supplementary material online, Figure S6). 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.

### 3.6 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 (see Supplementary material online, Figure S7). To investigate whether we have induced an oxLDL-specific T cell response, we therefore re-stimulated splenic leukocytes in vitro with oxLDL (5 \( \mu \)g/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 4).

### 3.7 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 h after stimulation of splenocytes with ConA. We observed a 75% reduction in IFN-\( \gamma \) production by splenic T cells from mice vaccinated with oxLDL-pulsed mDCs compared with T cells from mice treated with mDCs \( (P = 0.001) \) (Figure 5A). The level of IL-10 and IL-4 production by T cells was equal in both groups (Figure 5B and C, respectively).

### 3.8 Cellular response to vaccination strategy

To evaluate the effect of DC treatment on the numbers of different subsets of leucocytes, 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 leucocytes. We analysed the numbers of T cells (CD4 and CD8), regulatory T cells (CD4\(^+\)CD25\(^{hi}\)), and NKT cells (CD3\(^+\)NK1.1\(^+\)). We observed no significant differences in the numbers of blood leucocytes at any time point during the experiment (see Supplementary material online, Table S1). We also performed FACS analysis on leucocytes in spleen, liver, and mediastinal lymph nodes after sacrifice and again observed no differences between the various groups (data not shown).

### 3.9 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 >90% positive for the macrophage-specific markers F4/80 and CD11b, whereas they were <10% positive for the DC marker CD11c (see Supplementary material online, Figure S8A). 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 with the addition of serum from mice treated with mDCs (see Supplementary material online, Figure S8B), indicating that treatment with oxLDL-pulsed mDCs results in the formation of oxLDL-specific antibodies that reduce foam cell formation.

4. 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. 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. 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. Also, hyperlipidaemia suppresses the migration of skin DCs. 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 copper-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. Indeed, we showed that CCR7, necessary for migration towards lymphoid organs, is up-regulated in the mDCs 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, 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. Also, a recent paper by Packard et al. shows that foam cell formation as such does not affect the antigen-presenting capacity of DCs.

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 et al., a number of studies have shown the effectiveness of immunization against oxLDL or apoB-100 peptides in experimental animal models. Immunization leads to a reduction in atherosclerosis ranging from 40 to 70% in mice and rabbits. 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.10 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. 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 and co-workers33 nicely describes that initiation of atherosclerosis at different sites in the vasculature may involve different molecular pathways. 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 where 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 models36,37 and have been used to improve immunity in cancer38 and HIV-infected patients by inducing specific T cells.39,40 The attenuation of atherogenesis in our study was accompanied by an increase in T cell-dependent IgG antibodies towards Cuox-LDL and not towards MDA-LDL. This indicates that the treatment with oxLDL-pulsed mDCs induced an activation of oxLDL-specific T cells. Indeed, we show that spleen cells from mice treated with oxLDL-pulsed mDCs displayed an increased in vitro proliferation towards oxLDL when compared with 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 due 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 LDL−/− 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-β and IL-10. The induction of oral tolerance to

Figure 5 Cytokine production of splenocytes after in vitro stimulation. Total splenocytes were in vitro cultured with ConA during 24 h. Cytokine levels were determined in the supernatants (n = 5; ***P < 0.01; ****P = 0.001).
oxLDL and HSP60 was not accompanied by the induction of IgG or IgM specific for the orally administered antigen.44 These findings are in contrast to the present study where the treatment with oxLDL-pulsed mDCs induced anti-oxLDL antibodies, a reduction in IFN-γ 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 titres of oxLDL-specific IgG antibodies 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 and co-workers42 who showed that monoclonal IgG Fab antibodies directed to oxLDL blocked foam cell formation in macrophages. Also, Caligiuri et al.43 showed that sera from ApoE−/− mice, immunized with phosphorylcholine, reduced the uptake of oxLDL by macrophages. In addition, both Zhou et al.44 and Freigang et al.45 demonstrated an inverse correlation between lesion size and anti-MDA-LL IgG levels in mice immunized with MDA-LLD. 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-apoB-100 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.21,46

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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