The potential use of dendritic cells in mouse models of atherosclerosis

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Chapter 3

Immunomodulation with OCH-pulsed dendritic cells attenuates atherosclerosis

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Abstract

Natural killer T (NKT) cells contribute significantly to the inflammatory response in atherosclerosis. In atheroprone apolipoprotein E (apoE) deficient mice, NKT cells have an atherogenic phenotype and their activation with the synthetic ligand α-Galactosylceramide (α-GalCer) causes an increase in atherosclerotic plaque formation. Stimulation with the α-GalCer analog OCH is shown to provoke a T helper 2 (Th2) cytokine phenotype in NKT cells. In this study, we observed an increased interleukin-10 (IL-10) production after intraperitoneal treatment with OCH, but no effect on atherosclerosis. Therefore we used mature dendritic cells (mDCs) to deliver the OCH to the NKT cells in the liver. Treatment with OCH-pulsed mDCs resulted in an increased percentage of IL-10 producing NKT cells in the liver and in a subsequent reduction in atherosclerotic plaque formation. Additionally a reduction in cholesterol levels was observed in mice treated with OCH-pulsed mDCs. Altogether, this strategy of immunomodulation with mDCs loaded with OCH may form a new therapeutical approach to prevent atherosclerosis.

Introduction

In the chronic inflammatory response that underlies atherosclerosis, both innate and adaptive mechanisms are very important.[1] Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), are reported to initiate the autoimmune response by the uptake of autoantigens, such as oxidized low density lipoprotein (oxLDL) and heat shock proteins. Peptides of these antigens are presented to T cells via MHC class I and II molecules.[2, 3] By this, the peptides elicit the activation of T cells, especially T helper 1 (Th1) cytokine producing $CD4^+$ T cells.[4-7] T cell triggering naturally occurs within the lymphatic system, especially within the lymph nodes and spleen. After triggering, T cells migrate to the site of inflammation *i.e.* infiltrate the atherosclerotic plaque, where they may re-encounter their specific antigen, become activated and mediate their inflammatory damage. This is the onset of a process in which increasing numbers of immune cells are attracted to the atherosclerotic plaque and can result in occlusion of the vessels and severe cardiovascular disorders.

APCs such as macrophages and DCs are also attracted into the atherosclerotic plaque. APCs and especially the DCs express CD1 molecules, MHC class I like molecules, which specifically present lipid antigens to T cells.[8] In mice, only CD1d, a CD1 family member, is expressed on DCs[9] and Bobryshev et al. observed that CD1d is expressed within the atherosclerotic plaque.[10] The complex of CD1d with a lipid antigen can be recognized by invariant natural killer T (NKT) cells. NKT cells are a specialized subset of T cells expressing both an invariant T cell receptor (TCR) α chain composed of Vα14-Jα18 segments and the NK cell marker NK1.1. In atherosclerosis a colocalization of NKT cells and DCs within the shoulder regions of the plaque is observed, suggesting that NKT cells are activated by antigen presentation on the DCs.[11]

NKT cells can be activated using synthetic ligands such as α-galactosylceramide[9] (α-GalCer) and the α-GalCer analog ((2S,3S,4R)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-tetracosanoylamino)-1,3,4-nonanetriol) (OCH).[12, 13] Activation by α-GalCer induces a rapid aspecific mixed Th1/Th2 response, in which the NKT cells produce large amounts of IL-4, IL-10, IL-12, IL-13, and IFN-γ. Recent studies showed that in contrast with other Th1-mediated autoimmune-like diseases such as autoimmune diabetes,[14-16] experimental autoimmune encephalomyelitis[17, 18] and colitis,[19, 20] treatment with α-GalCer accelerated the disease process in atherosclerosis-prone apoE deficient mice.[21-23] OCH, which has a truncated sphingosine chain and therefore a lower affinity for CD1d, induces a more Th2-like cytokine profile. OCH-activated NKT cells produce predominantly IL-4, IL-10, and IL-13 and low levels of IFN-γ.[12, 13] This feature makes OCH an interesting glycolipid, which abrogates Th1-mediated immune responses.[12, 19, 24, 25]

Because of their capacity to stimulate T and NKT cells, DCs are widely used in vaccination therapies. DCs may be pulsed with an antigen *ex vivo* and subsequently these "pulsed" DCs are returned into the bloodstream. This approach is successfully used in cancer[26] and in several autoimmune diseases. DCs pulsed with bovine collagen type II protect mice from collagen-induced arthritis[27] and immature DCs (imDCs) pulsed with a peptide of glutamic acid decarboxylase protect nonobese diabetic (NOD) mice against type I diabetes.[28] In studies on cancer it was observed

that DCs pulsed with α-GalCer induced a prolonged IFN-γ-producing NKT cell response.[29, 30]

In this study we observed that intraperitoneal injections of OCH in low density lipoprotein receptor (LDLr) deficient mice had no effect on atherosclerosis despite an increased IL-10 production by splenic $CD4^+$ lymphocytes. In contrast, injection of OCH-pulsed mDCs in LDLr deficient mice reduced atherosclerotic plaque formation which may be explained by a significant increase in $IL\text{-}10^+$ producing NKT cells in the liver. Additionally a significant reduction in serum cholesterol levels was observed after treatment with OCH-pulsed mDCs.

Materials and 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 $LDLr^{-/-}$ and GFP^+ mice were obtained from Jackson's Laboratory and male C57BL/6j mice from Charles River Laboratories (Maastricht). $Jα281^{-/-}$ mice on a C57BL/6 background were obtained from Dr. M. Taniguchi. $Jα281^{-/-}LDLr^{-/-}$ double knockout mice were generated by crossing $Jα281^{-/-}$ mice with $LDLr^{-/-}$ mice. The offspring was crossed to produce mice with homozygous deletion in both LDLr and Jα281 genes. All mice were kept under standard laboratory conditions and bred in-house. The mice 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). Water and food were administered *ad libitum*.

Media and reagents

R1 cells, producing Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), and dendritic cells were cultured in IMDM (Cambrex, Belgium) supplemented with 8% FBS, 100 U/ml penicillin/streptomycin (both from PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μm β-mercaptoethanol (Sigma Aldrich, The Netherlands). β-GalCer was obtained from the Kirin Brewery Co Ltd, (Gunma, Japan). OCH ((2S,3S,4R)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-tetracosanoylamino)-1,3,4-nonanetriol) was synthesized as previously described by Fan et al. and dissolved in dimethyl sulphoxide (DMSO).[31] The synthesis of OCH was analyzed via nuclear magnetic resonance (NMR).

Intraperitoneal treatment with OCH

To test the direct effect of OCH on atherosclerosis, $LDLr^{-/-}$ mice were injected intraperitoneally with either 100 μg/kg β-GalCer (n=11) or OCH (n=13), twice a week for 7 weeks. Both β-GalCer and OCH were dissolved in PBS with 1% DMSO. After 3 weeks of Western-type diet feeding and prior to the treatment with β-GalCer and OCH, atherosclerosis was induced in both carotid arteries by bilateral perivascular collar placement as described previously.[32] The mice were fed a Western-type diet during the whole experiment (10 weeks). At the end of the experiment, mice were sacrificed and tissues were harvested after *in situ* perfusion with PBS and FormalFixx. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and

stored at -20°C until further use.

Treatment with OCH-pulsed mDCs

To test the effect of OCH-pulsed mDCs, bone marrow cells were harvested from the femur and tibia of C57BL/6j mice and were cultured for 10 days in complete IMDM supplemented with GM-CSF. Maturation of the DCs was accomplished via the addition of 1 µg/ml of LPS (from Salmonella Typhosa, Sigma Aldrich, The Netherlands) to the medium for 24 hours. Together with LPS, 100 ng/ml of OCH was added to a fraction of the DCs. Control DCs were incubated with LPS only. After 24 hours, the DCs were harvested and diluted in PBS. Subsequently, $LDLr^{-/-}$ mice were injected intravenously 8, 6 and 3 days before the mice were put on a Western-type diet. The mice received PBS (n=13), $1.5x10^6$ mature DCs (n=11) or $1.5x10^6$ OCH-pulsed mDCs (n=11). Next, the mice were either sacrificed one day after the last injection with DCs or the mice were fed a Western-type diet for 3 weeks and atherosclerosis was induced in both carotid arteries by bilateral perivascular collar placement as described previously.[32] Seven weeks after collar placement, the mice were sacrificed and tissues were harvested after *in situ* perfusion with PBS and FormalFixx. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use. In case of the $Jα281^{-/-}LDLr^{-/-}$ mice, mDCs and OCH-pulsed mDCs were administered and atherosclerosis was induced by feeding these mice a Western-type diet for 10 weeks without placement of perivascular collars.

Homing of injected DCs

To investigate the homing of injected DCs, bone marrow DCs were isolated from GFP^+ mice and were cultured for 10 days in presence of GM-CSF. Subsequently, the cells were maturated with LPS (1 µg/ml) and injected intravenously in $LDLr^{-/-}$ mice fed a normal chow diet or a Western-type diet 48 hours after injection, the mice were sacrificed and the distribution of GFP^+-DCs was determined via FACS-analysis of several organs. These organs were isolated and mononuclear cells were isolated using Lympholyte according to the manufacturer's protocol (Cedarlane, Hornby, Ontario, Canada).

Histological analysis

After sacrificing the mice, the carotid arteries were sliced (5 µm) proximal of the collar and the cryosections were stained with hematoxylin (Sigma Aldrich, The Netherlands) and eosin (Merck Diagnostica, Germany). Cryosections of the aortic root (10 µm) were stained with Oil-red-O and hematoxylin to determine plaque size. Corresponding sections of carotid arteries and aortic root were stained with a macrophage specific marker (MOMA-2, Research Diagnostic Inc., New Jersey) and a collagen specific marker (Masson's Trichrome, Sigma Aldrich, The Netherlands). All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Cholesterol and triglyceride levels

During the experiment, plasma samples obtained by tail vein bleeding were used to determine the total plasma cholesterol and triglyceride levels. Cholesterol

levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Triglyceride levels were quantified by using a ready-made kit (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard. Via SMART-analysis (3.2 x 30 mm, Smart System, Pharmacia) using the Superose 6 column the cholesterol distribution over different lipoproteins was analyzed.

Flow Cytometry

To check the maturation status of the DCs, cells were stained with the antibodies CD80-PE, CD86-FITC, CD40-PE, MHC II-PE, CD1d-FITC and CD11c-FITC (eBioscience, Belgium). In order to detect effects on cytokine production, 3 days after the last injection with β-GalCer or OCH, leukocytes were isolated from spleens using Lympholyte (Cedarlane, Canada). Per well of a 96-wells plate, $5x10^5$ of these mononuclear cells were stimulated for 24 hours with αCD3/CD28 (5 μg/ml) and 100 ng/ml OCH. Next the splenocytes were incubated with a leukocyte activation cocktail containing Golgi-stop (Becton Dickinson, CA). After 4 hours we performed intracellular staining as suggested by the manufacturer's protocol. In this experiment CD4-PerCP, IFNγ-APC, IL-10-PE and IL-4-FITC antibodies were used. To determine NKT cells in blood during DC treatment, blood was collected at several time points in EDTA-coated tubes. Red blood cells were lyzed using a lysis buffer containing 0.83% NH_4Cl in 0.01M Tris/HCL (pH 7.2). Subsequently the cells were stained with CD3-PerCP, NK1.1-FITC or PE-labeled α-GalCer loaded CD1d tetramer antibodies to detect NKT cells. To detect intracellular cytokines after the DC treatment, the mice were sacrificed one day after the last injection with DCs. Leukocytes were isolated using Lympholyte (Cedarlane, Hornby, Ontario, Canada) and CD3-PerCP, NK1.1-FITC, IL-10-APC and IFNγ-APC antibodies were used to detect intracellular antibodies in NKT cells. All antibodies were purchased from eBioscience (Belgium). Staining of the cells was done in PBS with 1% normal mouse serum. FACS analysis was performed on a FACSCalibur (Becton Dickinson, CA). Data were analyzed with Cell Quest software.

Antibody detection

Cuox-LDL was synthesized as described previously[33, 34]. MDA-LDL was made by addition of 0.5 M MDA to 10 mg of LDL for 3 hours at 37ºC. Antibodies against MDA-LDL and ox-LDL were determined according to Damoiseaux et al.[35] Briefly, maxiSorp 96 well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μg MDA-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.

Statistical analysis

Values are expressed as mean ± SEM. A two tailed Student's t-test was performed to compare data. When necessary a Mann-Whitney test was performed. Probability (*P)* values below 0.05 were considered significant.

Results

Effect of multiple intraperitoneal injections with OCH on atherosclerosis

To investigate the effect of OCH on atherosclerosis, $LDLr^{-/-}$ mice in which atherosclerosis was induced by Western-type diet feeding and perivascular collar placement around the carotid arteries, were used. Following collar placement, β-Galactosylceramide (β-GalCer) and OCH (100 μg/kg) were administered intraperitoneally twice a week for 7 weeks. The control, β-GalCer, has a β-linkage of galactose to the ceramide group and binds to CD1d but is not able to activate proliferation of and cytokine production by NKT cells.[9, 36] Although NKT cells are not completely inert to β-GalCer[36], β-GalCer is used as a control because it has no effect on atherosclerosis as shown by our group (unpublished results). After treatment, the mice were sacrificed and the degree of atherosclerotic plaque formation in β-GalCer-treated mice and OCH-treated mice was determined in the carotid arteries (Figure 1A and 1B, respectively) and in the aortic root (Figure 1D and 1E, respectively). Treatment with OCH had no significant effect on plaque size in both the carotid arteries (Figure 1C; 38,758±6,937 μm^2 versus 39,211±9,363 μm^2) or the aortic root (Figure 1F; 438,163±41,923 μm^2 versus 499,456±52,2998 μm^2) when compared with β-GalCer treated mice. During the experiment, total plasma cholesterol levels and body weight were not significantly different between both groups of mice (data not shown).

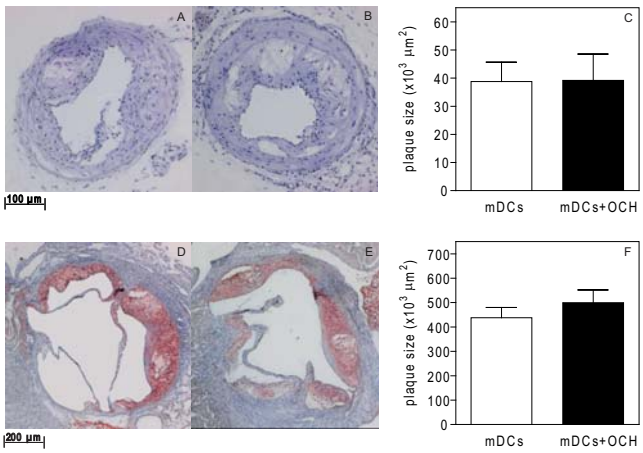

Figure 1. Effect of intraperitoneal injections with OCH on atherosclerotic plaque formation.
Atherosclerosis was induced in $LDLr^{-/-}$ mice by Western-type diet feeding and collar placement around both carotid arteries. Next, mice were treated with β-GalCer (n=11) or OCH (n=13) twice a week during seven weeks after which mice were sacrificed. Lesion size was determined in the carotid arteries of β-GalCer-treated (A) and OCH-treated mice (B) and in the aortic root of β-GalCer-treated (D) and OCH-treated (E) mice.

Multiple intraperitoneal injections with OCH influence the cytokine profile

OCH is reported to induce a Th2-like cytokine profile in NKT cells. Therefore we isolated spleens of β-GalCer-treated and OCH-treated mice 3 days after the last injection with β-GalCer and OCH and performed an intracellular FACS staining to determine the cytokine profile. Following restimulation with OCH *in vitro*, the percentage of IFN-γ producing cells within the $CD4^+$ population of the splenocytes decreased significantly with 34% from 1.20±0.14% in the β-GalCer-treated mice to 0.79±0.08% in the OCH-treated mice (Figure 2; $P<0.05$). Furthermore, a 76% increase in IL-10 producing cells within the $CD4^+$ population was observed (Online Figure 2; 0.76±0.14% versus 1.34±0.15%; $P<0.05$), while there was no effect on the percentage of IL-4 producing $CD4^+$ cells.

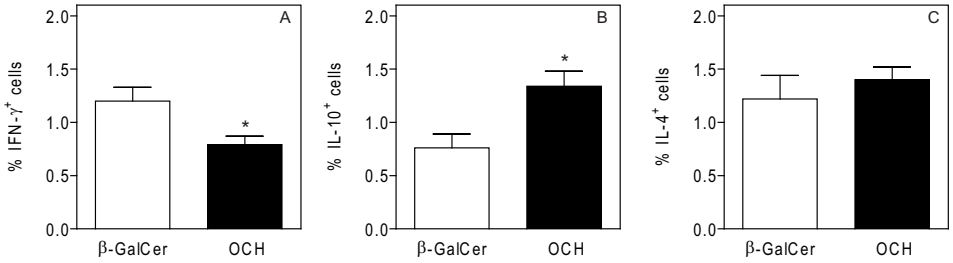

Figure 2. *In vivo* administration of OCH affects the cytokine profile within the spleen.

After multiple treatments with OCH, spleens of $LDLr^{-/-}$ mice fed a Western-type diet and equipped with collars around both carotid arteries were dissected and splenocytes were isolated. The splenocytes of β-GalCer-treated (n=5) and OCH-treated mice (n=6) were re-stimulated with αCD3/CD28 (5 μg/ml) and OCH (100 ng/ml) *in vitro*. After 24 hours the percentage of CD4+ T cells producing IFN-γ, IL-10 and IL-4 was determined via intracellular FACS analysis after gating for $CD4^+$ lymphocytes (*$P<0.05$).

OCH priming of DCs

In order to test the effect of OCH-pulsed mDCs on atherosclerosis, DCs had to be pulsed with OCH first. Therefore, bone marrow cells were isolated from the femur and tibia of C57Bl/6 mice and cultured for 10 days in presence of GM-CSF. To maturate the DCs, LPS (1 μg/ml) was added to the culture for 24 hours. The maturation status of the DCs was checked by FACS analysis. CD11c was constitutively expressed on all DCs *i.e.* imDCs, mDCs and OCH-pulsed mDCs. CD40, CD80 and CD86, well known maturation markers for DCs are upregulated after the addition of LPS. The peptide-antigen presenting molecule MHC class II was already present on imDCs and is only slightly upregulated due to maturation, while the lipid-antigen presenting molecule CD1d is upregulated due to the addition of LPS. OCH, when added together with LPS, had no additional influence on the maturation of the DCs (Figure 3).

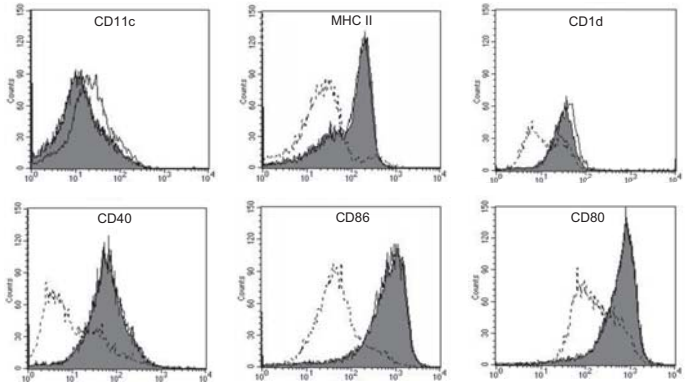

Figure 3. The effect of OCH on the maturation of DCs.
Bone marrow derived DCs were incubated with GM-CSF for 10 days. Subsequently, maturation was induced by LPS (1 µg/ml) in the presence or absence of OCH (100 ng/ml). After 24 hours expression of maturation markers was determined by FACS analysis. (dashed line: immature DCs, full black line:mature DCs, full black line with grey area: mature DCs pulsed with OCH).

Accumulation of i*ntravenously* injected mDCs in different organs

To investigate the homing of intravenously injected DCs, DCs were isolated from GFP$^+$ mice and maturated by the addition of LPS. Forty-eight hours after injection of mature GFP$^+$ DCs in chow diet fed and Western-type diet fed LDLr$^{-/-}$ mice, DCs were recovered in the lung, liver, spleen and lymph nodes around the heart (HLN). When fed a chow diet, 18.6% of the lymphocytes in the lung were GFP$^+$, while in the liver 3.8% was GFP$^+$. Relatively low percentages of the injected mDCs were found in spleen and lymph nodes (Figure 4A). When mice were fed a Western-type diet, 16.5% of the lymphocytes in the liver were GFP$^+$. The number of GFP$^+$ cells in other organs was not really different from that in the chow diet fed mice (Figure 4B). Taken into account the large number of leukocytes within the liver, we conclude that large numbers of the injected mDCs home to the liver.

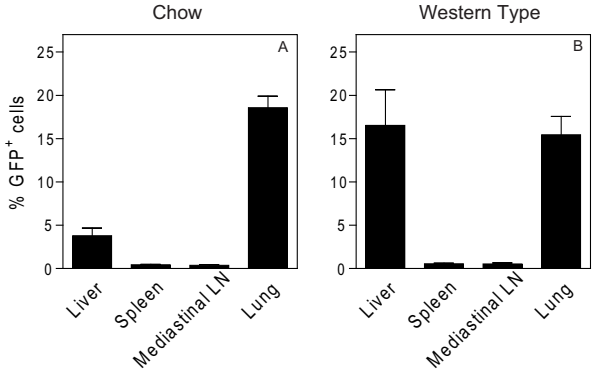

Figure 4. Homing of dendritic cells.
1.5x10^6 of mature GFP$^+$ DCs were administered intravenously to LDLr$^{-/-}$ mice fed a chow diet (A) or a Western-type diet (B) (n=4). After 48 hours the mice were sacrificed and the percentage of GFP$^+$ cells in organs were determined by FACS analysis

Repetitive injection of OCH-pulsed mDCs reduces atherosclerosis

To determine the effect of OCH-pulsed mDCs on atherosclerosis, PBS, mDCs and OCH-pulsed mDCs were administered 3 times in 8 days intravenously to LDLr$^{-/-}$ mice. After the treatment, atherosclerosis was induced by Western-type diet feeding and perivascular collar placement around both carotid arteries. Representative examples of hematoxylin-eosin stained atherosclerotic lesions in the carotid arteries of mDC-treated and OCH-pulsed mDC-treated mice are shown in Figure 5A and 5B, respectively. Injection of OCH-pulsed mDCs (9,400±2185 μm^2) resulted in a significant 70.6% reduction in plaque size in the carotid arteries when compared to mDC-treated mice (Figure 5C; 31,920±7,914 μm^2; P<0.05).

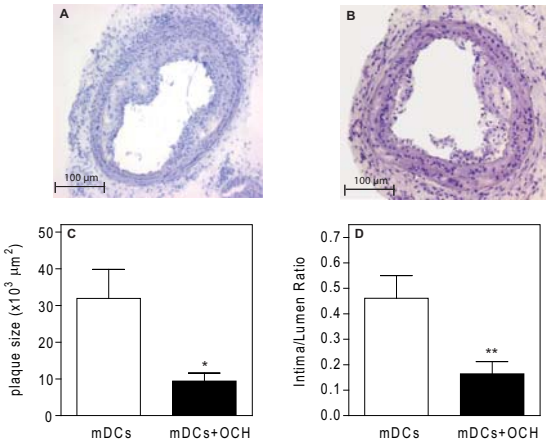

Figure 5. Immunomodulation with OCH-pulsed mDCs reduces lesion size in the carotid arteries.
LDLr$^{-/-}$ mice were three times injected with mDCs or OCH-pulsed mDCs prior to atherosclerosis induction. After a total of ten weeks on WT diet, mice were sacrificed and sections of the carotid arteries of mDC-treated mice (A, n=11) and OCH-pulsed mDC-treated mice (B, n=9) were stained with hematoxylin and eosin. Plaque size (C) and intima/lumen ratio (D) were determined (*P<0.05 **P<0.01).

Injection of OCH-pulsed mDCs (0.164±0.048) also reduced the intima/lumen ratio with 64.4% (Figure 5D; 0.461±0.089; P<0.01). Additionally, the treatment with mDCs did not differ from the treatment with PBS in plaque size (48,578±9,231 μm^2; P=0.20) and intima/lumen ratio (0.560±0.097; P=0.48) (not shown). A significant 58.1% reduction in plaque formation at the aortic root was observed when comparing OCH-pulsed mDC-treated mice (122,846±21,470 μm^2) with mDC-treated mice (Figure 6C; 293,181±36,193 μm^2; P<0.01). Representative Oil-red-O and hematoxylin stained examples of plaques at the aortic root of mDC-treated mice and mice treated with OCH-pulsed mDCs are shown in Figure 6A and 6B, respectively.

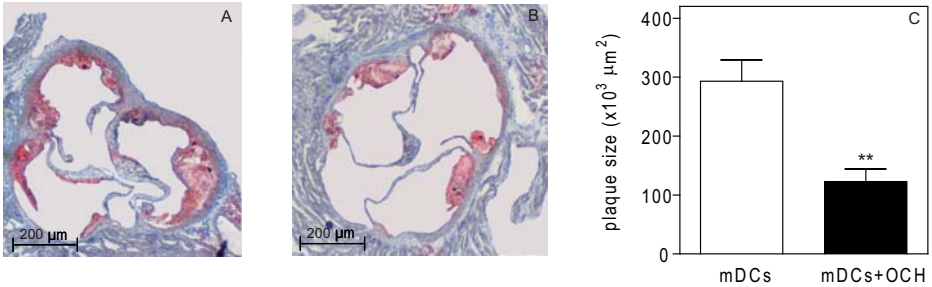

Figure 6. Effect of treatment on the lesion size in the aortic root.
LDLr$^{-/-}$ mice were treated with mDCs (A, n=11) or OCH-pulsed mDCs (B, n=9).Lesion size in aortic roots was determined after a total of ten weeks of WT diet feeding (C) (**P<0.01).

Plaque size at the aortic root of PBS-treated mice (228,667±32,747 μm^2) was again not significantly different from the plaque size in the mDC-treated mice (*P*=0.22, not shown). Both in plaques in the carotid arteries and at the aortic root no significant changes in plaque morphology were observed. Macrophages, smooth muscle cells and collagen were present to the same extent (data not shown). Because mDC-treatment and PBS-treatment gave similar results, only the mDC treatment was used as a control for further analysis.

To show that the effects of OCH pulsed mDCs are mediated by NKT cells, we performed a control experiment in mice lacking NKT cells ($J\alpha281^{-/-}$ mice) crossbred to a LDLr deficient background. Treatment of $J\alpha281^{-/-}/LDLr^{-/-}$ mice with OCH-pulsed mDCs had in contrast to the treatment of LDLr-/- mice no effect on plaque formation at the aortic root when compared with $J\alpha281^{-/-}LDLr^{-/-}$ mice treated with mDCs (Figure 7).

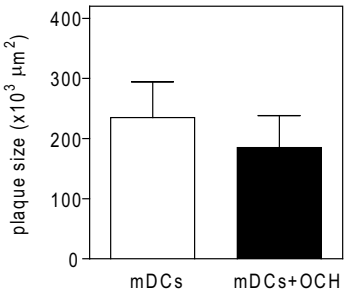

Figure 7. Effect of immunomodulation with OCH-pulsed mDCs on atherosclerosis in NKT cell deficient mice.
$J\alpha281^{-/-}LDLr^{-/-}$ mice were treated with mDCs or OCH-pulsed mDCs three times prior to atherosclerosis induction.

Increase in NKT cells due to vaccination with OCH-pulsed mDCs

During the experiment blood was withdrawn to determine the effect of vaccination with DCs on the number of NKT cells in blood. One day after the third vaccination with OCH-pulsed mDCs, which is 9 days after the start of the experiment, the percentage of NKT cells in the blood of mice treated with OCH-pulsed mDCs was significantly increased when compared with blood of mDC-treated mice (Figure 8A; 2.09±0.42% versus 0.94±0.24%, respectively; *P*<0.05). At the end of the experiment, the percentage of NKT cells in blood of mice treated with OCH-pulsed mDCs was still increased when compared to the mice treated with mDCs, but no significant difference was observed (1.18±0.14% versus 2.29±0.74%; P=0.20). To detect any changes in percentages of NKT cells in other organs we performed a new experiment, in which we repeated the treatment with the mDCs and OCH-pulsed mDCs as above but now sacrificed the mice one day after the last DC-injection. FACS analysis showed that treatment with OCH-pulsed mDCs significantly increases the percentage of NKT cells within the liver (6.62±0.79%) when compared with the mDC-treated mice (Figure 8B; 3.53±0.34%; *P*<0.05). No differences in NKT cell percentages were detected in other organs. The increase in NKT cell numbers in the circulation in mice treated with OCH-pulsed mDCs was not observed in mice treated with OCH intraperitoneally, indicating that treatment with DCs is more efficient in activating NKT cells.

The NKT cells in the liver of mice treated with OCH-pulsed mDCs also show a Th2-like cytokine profile. The percentage of IL-10 producing NKT cells increased 2.2-fold from 1.59±0.63% in mDC-treated mice to 3.42±0.33% in mice treated with OCH-pulsed mDCs (Figure 8C; $P<0.05$). In addition, the percentage of IFN-γ producing NKT cells in mice treated with OCH-pulsed mDCs (8.83±2.73%) was not significantly different from the percentage of IFN-γ producing NKT cells in mDC treated mice (6.71±2.52%; Figure 8D; $P=0.58$). No effect on the number of NKT cells and cytokine profile of NKT cells was observed immediately after the first and the second injection with OCH-pulsed mDCs when compared with mice treated with mDCS (data not shown).

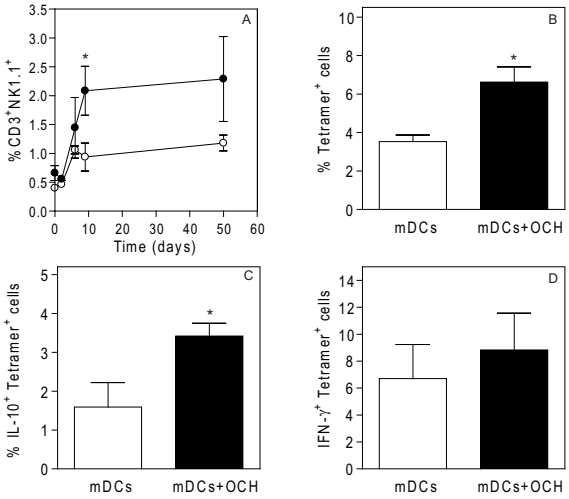

Figure 8. Effect of OCH-pulsed mDCs on the percentage and cytokine profile of NKT cells.
During the experiment, the number of NKT cells in blood was determined using FACS analysis (A). Grey line: mDC treated mice (n=3), black line: OCH-pulsed mDCs treated mice (n=3). In an independent experiment mice were sacrificed one day after the last injection with DCs and the percentage of CD1d-tetramer positive NKT cells in the liver was determined (B). The cytokine profiles of these NKT cells were determined using an intracellular FACS protocol. The production of IL-10 (C) and IFN-γ (D) was monitored (*$P<0.05$).

Vaccination with OCH-pulsed mDCs reduces cholesterol levels

During the *in vivo* DC vaccination experiment, body weight, serum cholesterol and serum triglyceride levels were measured at different time points. There was no significant difference in weight due to the treatment with OCH-pulsed mDCs (data not shown). Within the first 8 weeks of the DC-vaccination experiment, also no differences in total serum cholesterol levels were observed. However, between week 8 and 11 of the experiment a significant 23.7% lower cholesterol level was detected in mice vaccinated with OCH-pulsed mDCs (1,132±136 mg/dl) when compared with mDC-treated mice (Figure 9A; 1,483±67 mg/dl; $P<0.05$). To investigate whether this reduction in cholesterol is a reduction in cholesterol levels within a particular class of lipoproteins, a SMART analysis was performed. In serum of mice treated with OCH-pulsed mDCs, VLDL-cholesterol (111±21 mg/dl) and LDL-cholesterol (67±9 mg/dl) were significantly lower when compared with VLDL- and LDL-cholesterol levels in mDC-treated mice (Figure 9B; 188±17 mg/dl

and 104±9 mg/dl, respectively; $P<0.05$). HDL-cholesterol was also lowered but not significantly (46±12 mg/dl versus 25±2 mg/dl; $P=0.14$). No effects were observed on triglyceride levels in serum of these mice (data not shown).

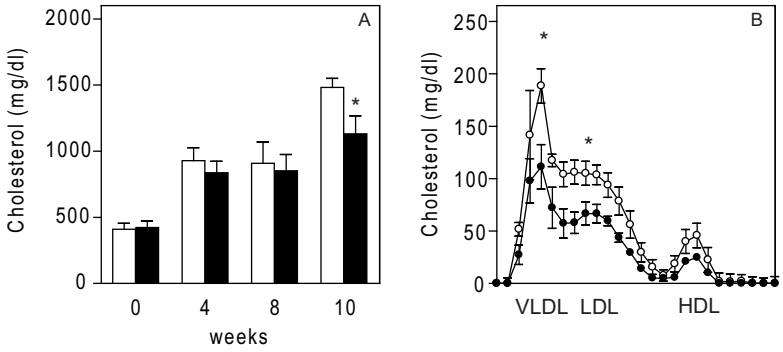

Figure 9. Effect of treatment with OCH-pulsed mDCs on cholesterol levels.
Serum cholesterol levels were measured at different time points (A). Sera were subsequently loaded onto a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles. (white = mDC; black = OCH-pulsed mDCs) (*$P<0.05$).

Vaccination with OCH-pulsed mDCs has no effect on antibody production

To investigate a possible role of the humoral immune response in reducing plaque formation, titers of antibodies specific for anti-modified LDL were determined. Treatment with OCH-pulsed mDCs had no influence on either malondialdehyde modified (MDA)-LDL and oxLDL-specific IgM and IgG titers in serum of the mice (Figure 10A and B, respectively).

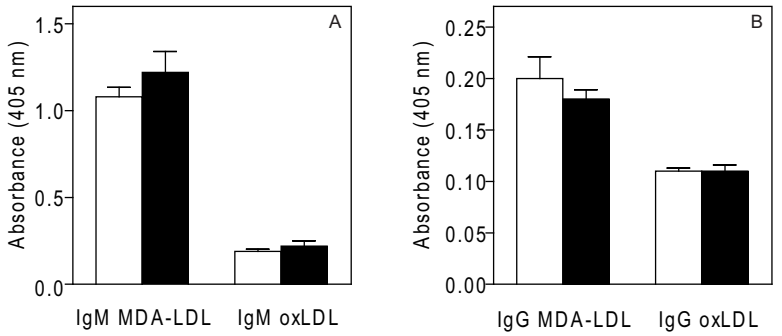

Figure 10. Influence of DC-treatment on MDA-LDL- and oxLDL-specific antibodies.
LDLr$^{-/-}$ mice were treated intravenously with mDCs (n=11) or OCH-pulsed mDCs (n=9) and at the end of the experiment serum levels of MDA-LDL- and oxLDL-specific IgM antibodies (A) and IgG antibodies (B) were measured using an ELISA. Values are mean OD 405nm ± SEM (white = mDCs; black = mDCs+OCH).

Discussion

The activation of antigen specific $CD4^+$ T cells is one of the main events in the inflammatory response of atherosclerosis. Within the atherosclerotic plaque, $CD4^+$ T cells are re-activated via the recognition of specific peptide-antigens presented by MHC class II molecules on APCs. Dendritic cells play a very important role in this process of antigen processing within the plaque. Normally, DCs are present in the intima of non-diseased arteries.[37] These DCs become either activated in early stages of atherosclerosis[10, 38, 39] while in more advanced stages, the DCs are recruited from the adventitia[40] and blood[41] into the lesion. Within the lesions, DCs are especially located in the rupture prone shoulder regions[11, 40] and are found to form cell clusters with T cells. Recently, co-localization of DCs with another T cell subtype, the NKT cell, was observed within the atherosclerotic plaque.[11] The colocalization of DCs and NKT cells in the shoulder regions of the plaque[38], and the observation of CD1d expression within the atherosclerotic plaque[10, 42] suggests that the DCs present a lipidic antigen via CD1d to the NKT cells resulting in their activation. These NKT cells, present in the atherosclerotic plaque[21, 22] were found to have an athero-promoting effect.[21-23]

The role of NKT cells in atherosclerosis has been studied by using the synthetic NKT cell ligand α-GalCer. Multiple intraperitoneal and/or intravenous injections of α-GalCer in atherosclerosis-prone apoE deficient mice resulted in a surprising aggravation of atherosclerosis due to the production of Th1 cytokines by NKT cells.[21-23] Especially, increased levels of IFN-γ were found in these treated $apoE^{-/-}$ mice.[21, 22] Studies on the effect of α-GalCer on other Th1-mediated diseases showed however that multiple injections with α-GalCer induced a more Th2-based cytokine profile of NKT cells.[14-20] Therefore it was surprising that treatment with α-GalCer resulted in an aggravation of atherosclerosis. Additionally, CD1d deficiency resulted in a reduction in atherosclerosis. $LDLr^{-/-}CD1d^{-/-}$ and $apoE^{-/-}CD1d^{-/-}$ mice showed a significant decline in plaque size when compared with $LDLr^{-/-}$ mice[22, 43] and $apoE^{-/-}$ mice[21, 23], respectively. From this we suggest that NKT cells in athero-prone mice are activated by certain endogenous ligands, which are still unknown and exert an athero-promoting effect. Stimulation with α-GalCer seems to worsen the situation. One explanation for this may be the increased production of IL-4, which is probably an athero-promoting Th2 cytokine.[44, 45]

In this study we used OCH, another synthetic NKT cell ligand. OCH has a lower affinity for CD1d because of a shorter lipid chain and due to this lower affinity, the TCR stimulation is shorter in time than with α-GalCer. Since IFN-γ production requires a longer TCR stimulation than IL-4 and IL-10, a more pronounced Th2 cytokine profile is induced after activation with OCH. Oki et al.[46] observed that the duration of NKT cell stimulation determines whether the NF-κB family member transcription factor c-Rel is transcribed effectively. c-Rel is identified as essential for IFN-γ production by NKT cells. They showed that c-Rel is transcribed in α-GalCer-stimulated, but not in OCH-stimulated NKT cells.[46]

In vivo administration of OCH in several mouse models is shown to abrogate Th1-mediated immune responses and to be protective against experimental autoimmune encephalomyelitis,[12] arthritis,[24] colitis[19] and diabetes.[25] In this study, OCH was administered intraperitoneally to $LDLr^{-/-}$ mice and an increased amount

of IL-10 producing cells was observed within the spleen. The increase in IL-10 was accompanied by a decrease in IFN-γ producing cells. This cytokine profile is in line with the studies on other Th1-mediated autoimmune diseases mentioned earlier. There was however no effect on the formation of atherosclerotic lesions induced by Western-type diet feeding and collar placement around the carotid arteries of $LDLr^{-/-}$ mice. Nakai et al.[22] showed before that injections of OCH accelerated the early phase of atherosclerosis. In that study, 8 weeks old $apoE^{-/-}$ mice, fed a regular chow diet were injected with OCH once within two weeks, three times in total. In this study they showed that the IFN-γ levels in serum of OCH-treated mice was lower than in serum of α-GalCer-treated mice while IL-4 levels were the same. This is however in contrast with other reports on OCH, because OCH is known for promoting Th2 cytokine production by NKT cells, especially when administered twice a week. Nakai et al[22], administered OCH once within two weeks and it is possible that this interval between two injections was too long to induce a Th2 profile. Additionally, data on IL-10 production are not presented. Another major difference between our study and the one of Nakai et al. is the used mouse model. Van den Elzen et al. showed that apoE is an important mediator in presentation of lipid antigens via CD1 molecules.[47] ApoE may bind exogenous lipid antigens and efficiently target them for receptor-mediated uptake by DCs. Furthermore, more important is apoE bound on the membrane to heparan sulfate proteoglycans, playing a role in the delivery of the lipid to compartments containing CD1d, leading to presentation of the lipid on CD1d. Since activation of NKT cells by OCH is CD1d dependent, apoE may have an important role in this process and the results obtained from $apoE^{-/-}$ mice may therefore be misleading. Additionally, our mice were fed a Western type diet instead of a regular chow diet. We hypothesize that this high fat diet may influence NKT cells before OCH was administered. This pre-activation of NKT cells may lead to another response to OCH, a more Th2 profile, than without this pre-activation. However, this pre-activation by a high fat diet needs further investigation.

Another explanation for the lack of an effect on atherosclerotic lesion development after injections with free OCH may be that the effect on cytokine levels we observed was very temporal. The mice were sacrificed within 48 hours after the last injection with OCH and the induced IL-10 production together with the decreased production of IFN-γ may have been not strong enough to abrogate the Th1 response against several autoantigens in atherosclerosis. A second explanation could be an ineffective and inefficient presentation of OCH by antigen presenting cells to the NKT cells in the liver after intraperitoneal injections.

We therefore used another way to administer OCH to $LDLr^{-/-}$ mice. In several studies, the usefulness of DCs as vaccination units to impair autoimmune diseases was shown. In our previous study, mDCs pulsed with oxLDL induced a protective oxLDL specific antibody response and a reduction in atherosclerotic plaque formation in $LDLr^{-/-}$ mice.[48] In a study by van Duivenvoorde et al. DCs were pulsed with collagen type II and this resulted in a reduction in arthritis due to a decrease in the collagen specific "Th1-associated" IgG2a response.[27] Additionally, Lo et al. showed that immature DCs pulsed with a peptide of glutamic acid decarboxylase protects NOD mice against type I diabetes.[28]

In our previous[48] and current study we show that mDCs injected intravenously,

particularly accumulate within the liver. Loading of dendritic cells with NKT cell ligands would therefore deliver the ligand directly to the largest pool of NKT cells in the body, *i.e.* the liver. In the liver, the DCs can present the ligand via CD1d and induce the activation of the NKT cells. When $LDLr^{-/-}$ mice were fed a Western-type diet, even more DCs accumulated within the liver, possibly due to the elevated inflammatory status in this organ.

It was shown before that NKT cells can be expanded *in vivo* via the administration of mature dendritic cells pulsed with α-GalCer.[30] This study, performed in humans, showed a more than 100-fold increase in circulating NKT cells in all patients treated with the pulsed mDCs. In two other studies a prolonged IFN-γ producing NKT cell response is induced after treatment with α-GalCer loaded mDCs.[29, 49]

Because OCH induces a Th2-cytokine production by NKT cells and DCs loaded with NKT cell ligands can induce a prolonged cytokine production by NKT cells, we used this technique to study the effect of OCH-pulsed mDCs on atherosclerosis. The maturation of DCs with LPS was not affected by the addition of OCH and the OCH-pulsed mDCs were injected in $LDLr^{-/-}$ mice. The treatment with OCH-pulsed mDCs resulted in a 70.6% decrease in atherosclerotic plaque size in the carotid arteries when compared with mDC-treated mice. The same extent of reduction was also observed at the aortic root of these mice (58.1% reduction). Additionally, this effect was absent in mice lacking NKT cells ($Jα281^{-/-}LDLr^{-/-}$ mice) showing that the observed effect is dependent on NKT cells. From these results we may suggest that the bad influence of NKT cells on atherosclerosis can be turned into an athero-protective effect, when the NKT cells are activated with OCH. The best way to deliver OCH is OCH loaded on DCs, because the drug itself was ineffective.

To investigate this athero-protective effect of NKT cells due to the DC treatment, the effects on NKT cell percentages and cytokine profile were determined. One day after the last injection with DCs, an increased percentage of NKT cells was observed in both blood and liver. In addition, more IL-10 producing NKT cells were found in the livers of mice treated with OCH-pulsed mDCs when compared with mDC-treated mice. The increased production of IL-10 and the unchanged production of IFN-γ could explain the reduced plaque size in these mice.[50] After activation within the liver, the NKT cells may possibly migrate out of the liver and become recruited to the atherosclerotic plaque. This migration to the plaque may explain the increased levels of NKT cells in blood after treatment with OCH-pulsed mDCs. Within the atherosclerotic plaque, the NKT cells may start excreting anti-atherogenic cytokines locally. These cytokines, such as IL-10, may influence the immune response in the vessel wall directly. It is shown before that IL-10 protects against atherosclerosis.[50-54] Another possibility is that the NKT cells may induce a bystander effect.[55, 56] It is known that the bystander effects induced by activation of NKT cells with α-GalCer and OCH are different. Upon stimulation with α-GalCer, NKT cells affect the functions of other cells such as T cells, B cells, NK cells and DCs in a direct or indirect manner. Little is known about the effect of OCH-activated NKT cells on neighbouring cells but recently Oki et al. demonstrated that OCH induces less production of IFN-γ and IL-12 by bystander cells due to a lower expression of CD40L on NKT cells.[57] A bystander effect on neighbouring cells such as macrophages and T cells within the plaque may contribute further to the abrogation of the Th1 inflammatory response of atherosclerosis.

Another bystander effect of NKT cell activation in the liver may also explain the observed reduction in cholesterol levels. During the experiment serum cholesterol levels were evaluated at several critical time points. Although there was no effect on the initial cholesterol levels, treatment with OCH-pulsed mDCs induced a significant 24% reduction in cholesterol levels after week 8 of the experiment. The reduced cholesterol levels were due to a lowering of VLDL- and LDL-cholesterol, while HDL-cholesterol was unaffected. This reduction can be caused by an effect on the activity of parenchymal cells in the liver. Von der Thüsen et al.[50] showed before that systemic IL-10 administration resulted in the lowering of VLDL and LDL cholesterol levels in $LDLr^{-/-}$ mice. In this study, the increased IL-10 production by the NKT cells in the liver may be responsible for the increased uptake of cholesterol from the blood and the subsequent secretion of cholesterol in the bile.[50]

To examine whether there are also some bystander effects of the activation of NKT cells via OCH-pulsed mDCs on the humoral immune response we determined the effect on oxLDL and MDA-LDL specific antibodies. There were however no effects on both IgM and IgG antibody titers.

In conclusion, the activation of NKT cells via the administration of OCH-pulsed mDCs reduces the atherosclerotic plaque formation in $LDLr^{-/-}$ mice. The reduction in atherosclerosis can be explained by an increased percentage of IL-10 producing NKT cells in the liver. This IL-10 in the liver can induce a bystander effect that may be responsible for the lowering of cholesterol levels in the treated mice. Altogether, this strategy of immunomodulation with mDCs loaded with OCH could be used as a new therapeutical approach to prevent atherosclerosis.

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