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## The potential use of dendritic cells in mouse models of atherosclerosis

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# Chapter 4

**Adoptive transfer of immature dendritic cells prevents progression of established atherosclerosis in LDLr<sup>-/-</sup> mice**

*-Manuscript submitted-*

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### **Abstract**

Dendritic cells have the unique capacity to initiate and, more importantly, to regulate the immune response. Due to these properties, they have been used as an immunotherapy in many different models for cancer and autoimmune diseases. Over the past years, there has been accumulating data identifying an important role for the immune system and inflammation in atherosclerosis. Therefore, in this study we explored the possibility to use dendritic cells in a mouse model of established atherosclerosis to modulate disease progression. For this end male LDLr<sup>-/-</sup> mice were fed a Western-type diet for 20 weeks. After 20 weeks of diet, a baseline group of mice was sacrificed and others received three *i.v.* injections with either PBS or ImDCs (n=10; 1.5×10<sup>6</sup> cells per injection). Subsequently, these mice were kept on diet for another 10 weeks. Upon sacrifice, lesion size was determined. Plaque area analysis at different sites throughout the vasculature showed that plaque progression during the additional 10 weeks on diet was inhibited in mice treated with ImDCs. During the same time, plaque size in the PBS-treated group continuously increased. The injection of ImDCs led to an increased peripheral/intrahepatic CD4/CD8 ratio and an induction of regulatory T cells. In addition, ImDCs treatment affected the cholesterol metabolism by reducing LIGHT and Lymphotoxin- $\alpha$  which resulted in lowered cholesterol and triglyceride levels. Furthermore, treatment resulted in a more beneficial lipoprotein profile with reduced VLDL and increased HDL levels. We also observed an increase in CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells after injection of ImDCs but adoptive transfer of these cells in our current model of established atherosclerosis did not affect lesion size. Our data demonstrate the immunomodulatory potential of ImDCs in a model for established atherosclerosis and further supports the possibility to use dendritic cell-based treatment in cardiovascular disease.

## Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells and have the unique ability to activate naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells during the primary phases of an immune response. Immature DCs (ImDCs) patrol the periphery and sample their micro-environment. In the steady state, ImDCs will take up self-antigens but remain in their immature or semi-mature state. These cells lack the appropriate expression of costimulatory molecules, such as CD80 and CD86. Therefore, presentation of self-antigens to T cell will lead to their anergy or to the induction of IL-10 producing regulatory T cells, hence, resulting in tolerance.<sup>1</sup> In the case of an infection, DCs sense “danger signals” and become fully matured and are able to activate T cells.<sup>2</sup> In healthy arteries, scattered DCs reside in the sub-endothelial space as well as in the media.<sup>3</sup> Arterial DCs may play an important role in monitoring the artery for antigens. During lesion development, lesions become enriched in DCs with a more mature phenotype. Lesional DCs may play an important role in activating T cells and thus sustaining plaque inflammation. In addition, DCs can also regulate atherosclerosis via secretion of various cytokines, such as IL-12 or IL-10.<sup>4</sup> These unique characteristics make DCs an interesting candidate for immunotherapies. Additionally, by modifying autologous DCs *ex vivo* in either tolerogenic or activating DCs, one can optimize their properties for further therapeutic applications. Indeed, DCs have been used in many animal models and in cancer therapy as an immunotherapy.<sup>5, 6</sup> Furthermore, DCs are already being used in clinical trials to activate tumor-specific CD8<sup>+</sup> cells.<sup>7</sup> Recently, we described the use of oxidized LDL (oxLDL)-pulsed DCs in a vaccination protocol in LDLr<sup>-/-</sup> mice. We showed that injection of oxLDL-pulsed DCs prior to the induction of atherosclerosis induced an oxLDL-specific humoral response, which leads to protection against atherosclerosis.<sup>8</sup> Because of the tolerogenic features of immature DCs (ImDCs), we hypothesized that the use of ImDCs could be a simple way to introduce a protective immunomodulation of established atherosclerosis. It has been shown that ImDCs can expand the regulatory T cell population (Tregs) by the production of IL-10<sup>9, 10</sup> and there is accumulating evidence suggesting a protective role of Tregs in atherosclerotic lesion development and progression.<sup>11, 12</sup> Furthermore, the injection of immature DCs has been shown to induce tolerogenic cells that play a protective role in a number of auto-immune diseases.<sup>13-15</sup> Therefore, we have explored the potential of immature DCs as an immunotherapy in a model of established atherosclerosis. In the present study we show that adoptive transfer of unloaded ImDCs *i.v.* into LDLr<sup>-/-</sup> mice with established lesions prevents further plaque progression at various sites of lesion development. The inhibited lesion progression may be explained by the induction of tolerance and the reduced mRNA levels of LIGHT and lymphotoxin- $\alpha$  which are correlated to the observed lowered levels of cholesterol. Consequently, our current data shows for the first time the beneficial effect of treatment of established atherosclerosis with immature dendritic cells, which further underlines the necessity to clinically explore immunomodulation by using dendritic cells.

## Materials and methods

### Media and reagents

Cell culture medium was IMDM (PAA, Germany) supplemented with 8 % FCS (PAA, Germany), 100 U/ml streptomycin/penicillin (PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20  $\mu$ M  $\beta$ -mercaptoethanol.

### 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<sup>-/-</sup> mice were from Jackson Laboratories on a C57BL/6 background and bred in-house. Male C57BL/6J mice were from Charles River Laboratories. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 10-12 weeks old at the start of the experiment. Diet and water were administered *ad libitum*.

### Generation and injection of BM-DCs

Bone-marrow cells of C57BL/6 mice were harvested and cultured during 10 days in complete IMDM in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) to obtain immature DCs. Purity was assessed by flow cytometry using CD11c as a specific DC marker (purity > 90%).

### Induction of atherosclerosis

Male LDLr<sup>-/-</sup> mice were put on a western-type diet to induce atherosclerosis. After 20 weeks of diet, the baseline group was sacrificed (n=10). Remaining mice then received 3 *i.v.* injections (1 injection every other day) of ImDCs (1.5x10<sup>6</sup> cells in 100  $\mu$ l; n=10) or 100  $\mu$ l PBS (n=10) and were kept on western-type diet for an additional 10 weeks. After a total of 30 weeks on diet, mice were sacrificed. Tissues were then harvested after *in situ* perfusion using PBS and subsequent formalin perfusion. Fixated hearts and aortas were stored in formalin, while the brachiocephalic arteries 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

To determine plaque size, cryosections of the aortic root (10  $\mu$ m), brachiocephalic artery (5  $\mu$ m) and aortic arch (5  $\mu$ m) were stained with Oil-Red-O and hematoxylin (Sigma Aldrich, The Netherlands). 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). For the heart valves, the largest plaque and four flanking sections were used for assessment. The entire aorta was isolated and photographed. Relative plaque area was assessed *en face* by calculating the ratio of atherosclerotic lesions (white) to the surface of the entire aorta. All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

### **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).

### **Flow Cytometry**

After sacrifice, the blood, spleen, liver and mediastinal lymph nodes were isolated (n=5 per group). Single cell suspensions were obtained by using a 70  $\mu$ m cell strainer (Falcon, The Netherlands). Blood and spleen cells were lysed using 0.83%  $\text{NH}_4\text{Cl}$  in 0.01 M Tris/HCl pH 7.2. Mononuclear cells from the liver were isolated from non-parenchymal cells using Lympholyte (Cederlane, Canada). Subsequently 300.000 cells were stained with antibodies (Ebioscience, Belgium). FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software.

### **ELISA for detecting IgG and IgM**

Total and oxLDL-specific IgM, IgG, IgG1 and IgG2c levels were detected using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Francisco, USA), according to manufacturer's instructions. Briefly, for the detection of total antibody levels serum samples were 1:1 diluted in PBS and absorbance was read at 405 nm. For the detection of oxLDL-specific Ab, high binding plates were first coated overnight with 50  $\mu$ l Cuox-LDL (5  $\mu$ g/ml). Plates were then washed and 50  $\mu$ l of diluted serum samples were added. Absorbance was read at 405 nm.

### **ELISA for detecting cytokines**

Spleens from mice treated with ImDCs (n=5) or PBS (n=5) were isolated and smashed through a 70  $\mu$ m filter. Red blood cells were lysed using 0.83%  $\text{NH}_4\text{Cl}$  in 0.01 M tris/HCL, pH 7.2. Splenocytes were cultured in triplicates at  $2 \times 10^5$  cells/well in the presence or absence of oxLDL (5  $\mu$ g/ml) in complete RPMI 1640 (cRPMI). Concanavalin A (ConA, Sigma-Diagnostics, MO) (2  $\mu$ g/ml) served as positive control. After 24 hours, cytokine levels in the supernatant were determined, according to the manufacturer's protocol (Ebioscience, Belgium). Levels of TGF- $\beta$ , IFN- $\gamma$ , IL-10 and IL-4 were measured in the supernatants of the ConA stimulated spleen (Ebioscience, Belgium). In short, spleen culture triplicates treated with ConA (n=5 per group) were pooled and cytokine levels were determined in the undiluted supernatant following the manufacturer's instructions.

### **Adoptive transfer of CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells**

8 male LDLr<sup>-/-</sup> mice on a western-type diet were injected with ImDCs as described above. Three days after the last injection, mice were sacrificed and lymphocytes were isolated from the spleen and liver using a density gradient (Lympholyte, Cederlane). CD4<sup>+</sup>DX5<sup>+</sup> cells were isolated using CD4-FITC (Ebioscience, Belgium) and the Anti-FITC MultiSort Kit and subsequently CD49b (DX5) Microbeads,

following manufacturer's protocol (both from Miltenyi, The Netherlands). Purity of the isolated population was assessed by flow cytometry. A single *i.v.* injection of PBS, CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>-</sup>, or CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells (150.000 cells in 100  $\mu$ l) was given to mice with established atherosclerosis. Mice were sacrificed after 10 more weeks of diet feeding.

### Real-Time PCR

mRNA was isolated from livers using the guanidium isothiocyanate (GTC) method and reverse transcribed using RevertAid M-MiLV reverse transcriptase. Quantitative gene expression analysis was performed on the ABI7300 using sybergreen technology. The expression was determined relative to the average expression of household genes (36B4, 18S,  $\beta$ -actin and GAPDH). Expression levels of mice treated with PBS were set to 1.

**Table 1: Primer sequencens**

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
18s	CCATTCGAACGTCTGCC	GTCACCCGTGGTCACCATG
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
$\beta$ -actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
GAPDH	TCCATGACAACCTTGGCATTG	TCACGCCACAGCTTTCCA
Hepatic Lipase	CAGCCTGGGAGCGCAC	CAATCTTGTCTTCCCGTCCA
ABCA1	GGTTTGGAGATGTTATACAATAGTTGT	TTCCGGAAACGCAAGTC
ABCG1	AGGTCTCAGCCTTCTAAAGTTCCTC	TCTCTCGAAGTGAATGAAATTTATCG
Lipoprotein Lipase	CCAGCAACATTATCCAGTGCTAG	CAGTTGATGAATCTGGCCACA
LIGHT	TGGAGCCCGTTACTACTATGTG	TTCCGGTAGCGGGATGTG
Lymphotoxin- $\alpha$	TGGAGCCTTTGCACTGTAGATTC	CGTGAGAGCTCCAGGTTATTTAGT
Lymphotoxin- $\beta$ receptor	AATTATGGATACTGACGTCAACTGTGT	CCCTGGATCTCACATCTGGTATG

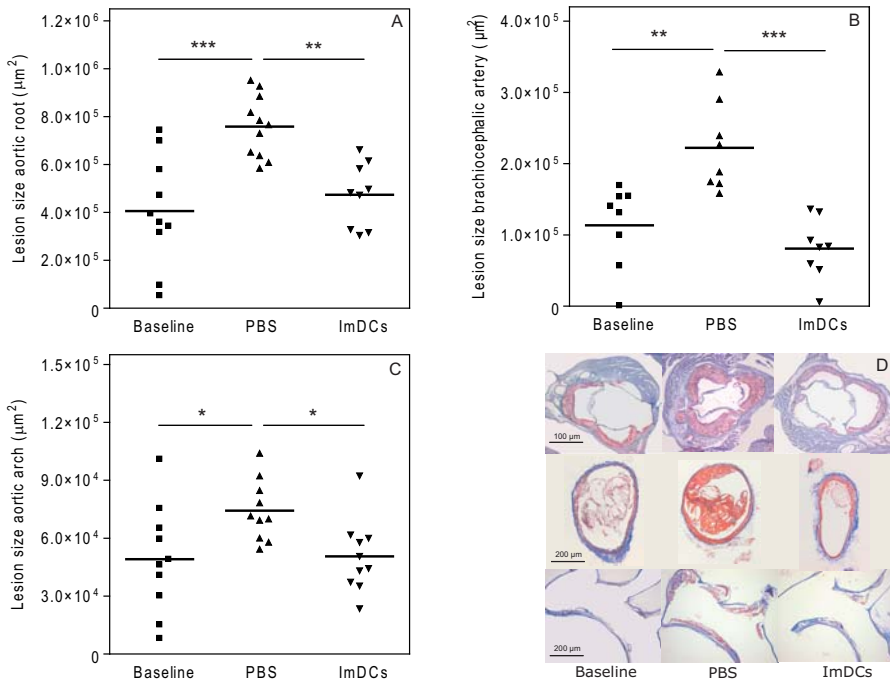
### Statistical analysis

Values are expressed as mean  $\pm$  SEM. Data were analyzed with either a parametric or non-parametric ANOVA when comparing three groups followed with Turkey post T-testing. When comparing two groups, a two-tailed Student's T-test or Mann-Withey U test has been used. Statistical analysis was performed using the InStat3 software. Probability values of  $P < 0.05$  were considered significant.

## Results

### Injection of ImDCs prevents progression of atherosclerosis

To induce established atherosclerosis,  $LDLr^{-/-}$  mice were put on a western-type diet for 20 weeks. At this time-point, the mice of the baseline group were sacrificed and the remaining mice received three *i.v.* injections of either ImDCs or PBS. After a total of 30 weeks of diet feeding, mice were sacrificed and plaque formation was analyzed at different sites in the vasculature. Figure 1A shows representative pictures of plaque size in the aortic root. Continuous feeding of Western-type diet resulted in a significant 1.5-fold increase in plaque size in mice that were treated with PBS compared to the baseline group ( $4.06 \times 10^5 \pm 7.22 \times 10^4 \mu\text{m}^2$  vs  $7.59 \times 10^5 \pm 3.88 \times 10^4 \mu\text{m}^2$ ), while plaque progression in mice treated with ImDCs was completely prevented ( $4.74 \times 10^5 \pm 4.74 \times 10^4 \mu\text{m}^2$ ) ( $P=0.0118$ ). Treatment with immature DCs had the same effect in the brachiocephalic artery (Baseline:  $1.13 \times 10^5 \pm 2.05 \times 10^4 \mu\text{m}^2$ ; PBS:  $2.22 \times 10^5 \pm 2.16 \times 10^4 \mu\text{m}^2$ ; ImDCs:  $8.08 \times 10^4 \pm 1.5 \times 10^4 \mu\text{m}^2$ ;  $P=0.0001$ ) (Figure 1B). Plaque size in the aortic arch was also determined and we again observed an attenuation of plaque progression (Baseline:  $4.91 \times 10^4 \pm 8.84 \times 10^3 \mu\text{m}^2$ ; PBS:  $7.42 \times 10^4 \pm 5.01 \times 10^3 \mu\text{m}^2$ ; ImDCs:  $5.06 \times 10^4 \pm 6.01 \times 10^3 \mu\text{m}^2$ ) ( $P=0.0241$ ) (Figure 1C). Representative pictures of the different sites are depicted in Figure 1D.

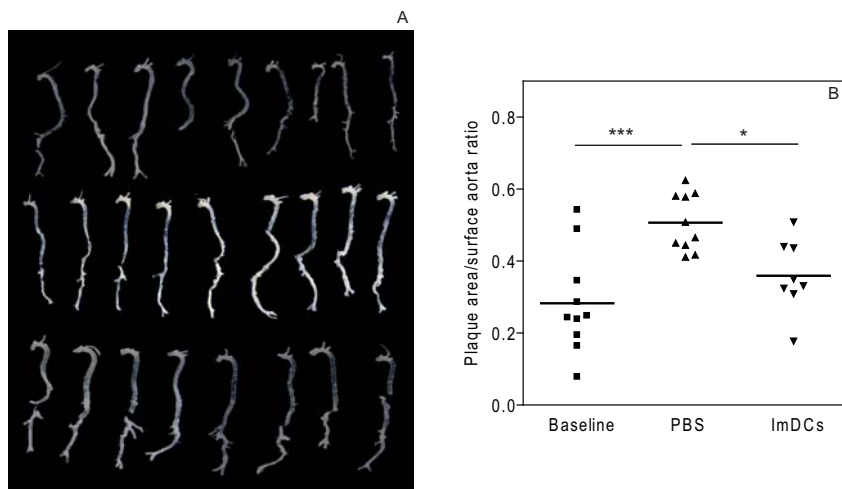


**Figure 1: Adoptive transfer of ImDCs prevents further progression of plaque size throughout the vasculature**

$LDLr^{-/-}$  mice were put on a western-type diet for 20 weeks. At this time point, the baseline group was sacrificed and other mice were given three *i.v.* injections of PBS or ImDCs ( $1.5 \times 10^6$  cells in  $100 \mu\text{l}$ ). After a total of 30 weeks of diet feeding, mice were sacrificed and lesion size was determined in the aortic root (A), the brachiocephalic artery (B) and the aortic arch (C). Representative pictures of lesion size at the different sites are shown in D (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

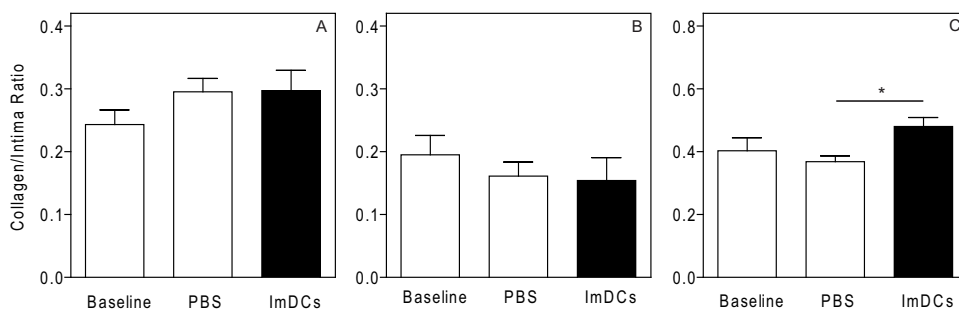


In addition, when determining the relative plaque size in the entire aorta *en face*, we observed the same effect: mice that received PBS injections showed a 1.8-fold significant increase in plaque area after 30 weeks of diet compared to mice in the baseline group ( $0.283 \pm 0.045$  vs  $0.507 \pm 0.025$ ). However, mice that received ImDCs showed no plaque progression ( $0.359 \pm 0.036$ ) ( $P=0.0005$ ) (Figure 2A and 2B). We then assessed plaque morphology by the number of macrophages and T cells in the aortic valves. Relative numbers of macrophage and T cell numbers were not affected (data not shown). Collagen content was assessed at all sites but was only significantly changed in the aortic arch of mice treated with ImDCs (Figure 3C).



**Figure 2: Plaque size growth in the entire aorta was prevented after ImDCs treatment**

Entire aorta's were isolated and photographed after PBS and fixative perfusion. Representative pictures of the aorta of baseline, PBS and ImDCs treated mice are shown in A. The plaque area/surface aorta ratio was quantified (B) ( $*P<0.05$ ;  $***P<0.001$ ).

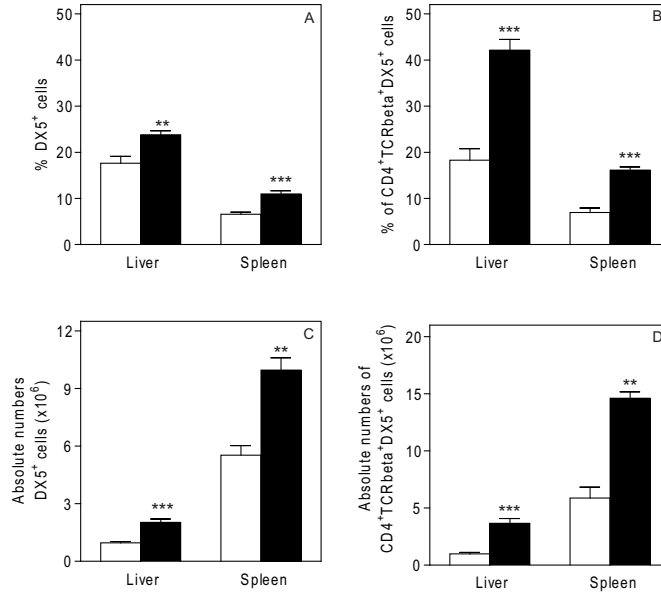


**Figure 3: Collagen content was only affected in the aortic arch**

Plaque composition was assessed in the aortic root (A), the brachiocephalic artery (B) and the aortic arch (C). The amount of collagen was determined by quantifying the ratio of collagen to intima size and was only increased in the aortic arch ( $*P<0.05$ ).

**Effect of adoptive transfer of ImDCs on lymphocyte subsets**

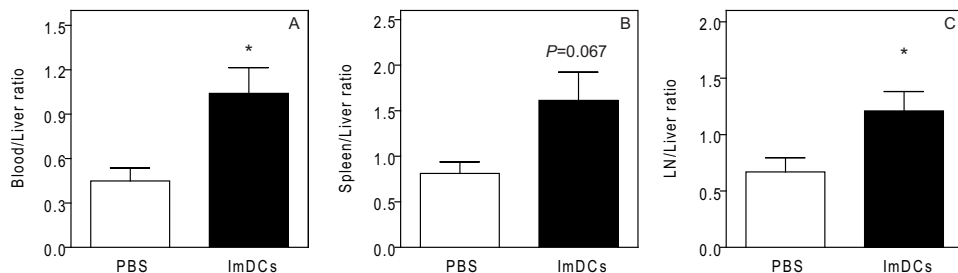
To evaluate the effect of ImDCs injections *in vivo*, we analyzed at 3 days, 3 weeks and 10 weeks after injection the cellular composition of spleen, liver, lymph nodes and blood by flow cytometry. At three days after the last injection of ImDCs, we observed an increased percentage of CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells in both the liver (18.30±2.47% vs 42.15±16.12%  $P<0.001$ ) and spleen (6.97±0.97% vs 16.12±0.71%  $P<0.001$ ) compared to PBS treatment (Figure 4).



**Figure 4: Induction of CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells 3 days after injection of ImDCs in both liver and spleen**

LDL<sup>-/-</sup> mice received three *i.v.* injections of PBS (white) or ImDCs (1.5x10<sup>6</sup> cells; black) and were sacrificed three days after the last injection. The percentage and absolute numbers of DX5<sup>+</sup> (A and C, respectively) and CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> (B and D, respectively) were determined in liver and spleen by flow cytometry (\*\*= $P<0.01$ ; \*\*\*= $P<0.001$ ).

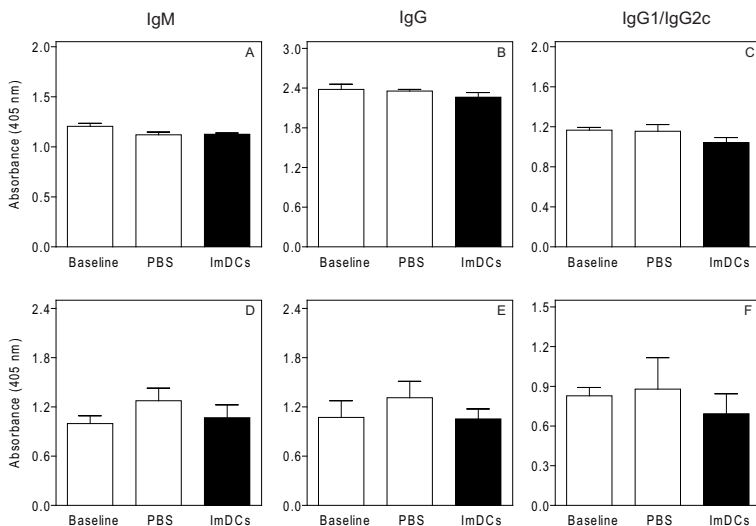
At this time point we also observed an increase in the numbers of CD11c<sup>+</sup> cells in the circulation (6.45±0.63% vs 10.77±0.87%;  $P=0.0037$ ) and a decreased CD4/CD8 ratio in blood (0.89±0.04 vs 0.69±0.07;  $P=0.047$ ), lymph nodes (1.35±0.03 vs 1.10 ±0.06;  $P=0.006$ ) and liver (1.95±0.42 vs 0.86±0.12;  $P=0.024$ ). In addition, we observed an increased peripheral/intrahepatic ratio of CD4/CD8 indicative of CD8 trapping in the liver (Figure 5). Three weeks after injection of the ImDCs, the early increase in CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells could no longer be observed in liver and spleen (data not shown). However, there was still an increase in the number of CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells (1.26±0.05% vs 2.41±0.39%;  $P=0.049$ ) and CD11c<sup>+</sup> cells in the mediastinal lymph nodes (3.75±0.22% vs 4.73±0.31;  $P=0.047$ ). Additionally, we also observed an increase in CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cells in blood (0.25±0.08% vs 0.71±0.12%;  $P=0.021$ ), and this increase was still present at 10 weeks after injection (0.95±0.05% vs 1.55±0.24%;  $P=0.049$ ).



**Figure 5: Increased peripheral/intrahepatic CD4/CD8 ratio after ImDCs treatment is indicative of tolerance induction**

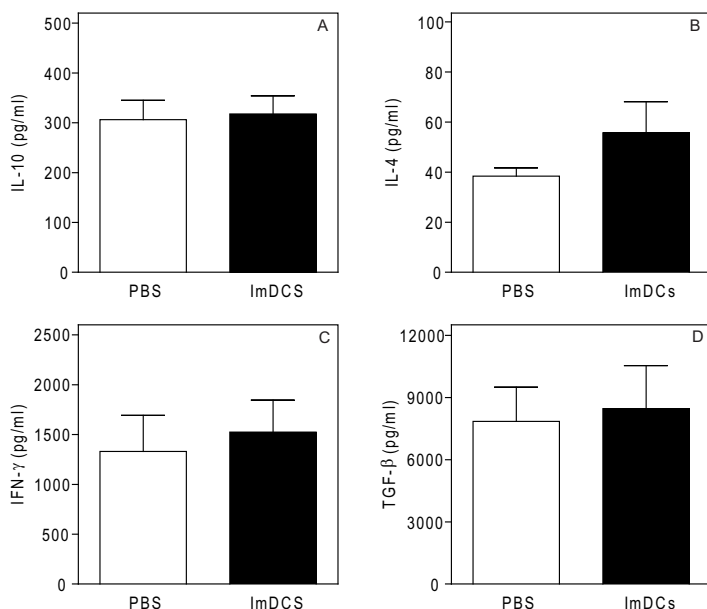
LDL<sup>r/-</sup> mice on a WT-diet were given 3 *i.v* injections of ImDCs (1.5 x10<sup>6</sup> cells in 100 µl). Mice were sacrificed 3 days after injection and the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was determined in different organs by means of flow-cytometry. Subsequently we calculated the peripheral/intrahepatic CD4/CD8 ratio for blood (A), spleen (B) and lymph nodes (C). The increased peripheral/intrahepatic ratio suggests CD8<sup>+</sup> T cell trapping in the liver, a key process in tolerance induction (\**P*<0.05).

To further test whether the adoptive transfer of ImDCs affected the humoral and T cell response, we checked for antibodies and T cell proliferation. Plasma samples from each mouse were obtained after sacrifice and total IgG, IgM and subclass specific antibodies were determined. Also, IgG, IgM and subclass specific antibodies against oxLDL were evaluated. We observed no differences in total IgM, IgG, and IgG1/IgG2c ratio (Figure 6A-C); and in oxLDL-specific IgM, IgG, and IgG1/IgG2c ratio (Figure 6D-F). Additionally, injection of ImDCs did not lead to altered proliferation or cytokine production after *ex vivo* stimulation of splenic T cells (Figure 7).



**Figure 6: Humoral response was not affected after adaptive transfer of ImDCs**

Serum of mice with established lesions was isolated 10 weeks after injections and the amount of antibodies was determined. Serum was 1:1 diluted in PBS and total IgM (A), IgG (B) and IgG1/IgG2c ratio (C) was calculated. Also the oxLDL-specific IgM (D), IgG (E) and IgG1/IgG2c ratio (F) was determined but no differences were observed. Absorbance was read at 405 nm.

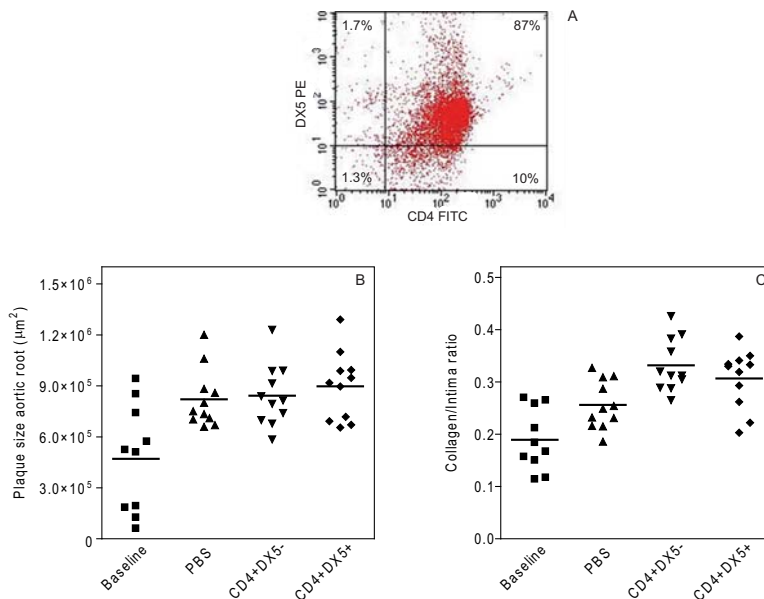


**Figure 7: Cytokine production of splenocytes after *in vitro* stimulation was not affected**

Supernatants of total splenocytes activated with ConA were collected and the level of IL-10 (A); IL-4 (B); IFN- $\gamma$  (C) and TGF- $\beta$  (D) were determined.

### **The inhibition of plaque progression after injection of ImDCs does not result from increased numbers of CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells**

Because of the therapeutic potential of tolerogenic DX5 cells in models for rheumatoid arthritis and multiple sclerosis, we determined whether the inhibition of plaque progression induced by ImDCs was mediated by CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells. To this end, we isolated CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells from the spleen and liver 3 days after ImDCs injections and transferred these cells into recipient LDLR<sup>-/-</sup> mice with established atherosclerosis. After 10 additional weeks on Western-type diet, mice were sacrificed and lesion progression was assessed. Plaque size in the aortic root did not increase during the ten extra weeks of diet in the PBS treated group by a factor 1.74. Treatment with CD4<sup>+</sup>DX5<sup>+</sup> cells did not affect lesion progression compared to the PBS treated group. Also collagen content was not affected after treatment (Figure 8). The additional control group treated with CD4<sup>+</sup>DX5<sup>-</sup> cells did also demonstrate the same plaque progression as the PBS or CD4<sup>+</sup>DX5<sup>+</sup> treated animals compared to baseline.

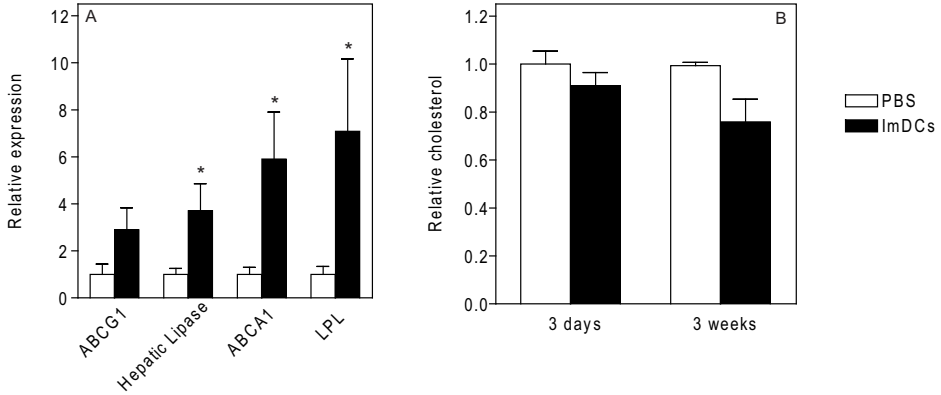


**Figure 8: Adaptively transferring CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells does not prevent plaque progression in a model of established atherosclerosis**

8 LDLr<sup>-/-</sup> mice on a western-type diet were injected three times with ImDCs. Three days after the last injection, mice were sacrificed and spleen and liver were isolated. CD4<sup>+</sup> cells from both liver and spleen were isolated using CD4-FITC beads. After detaching the beads, DX5<sup>+</sup> cells were positively selected using DX5 beads. CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> obtained from spleen and liver were pooled and purity was assessed with flow-cytometry (A). LDLr<sup>-/-</sup> mice with established atherosclerosis received a single *i.v.* injection of either CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup>, CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>-</sup> cells or PBS (150,000 cells in 100 µl) and after 10 more weeks of western-type diet feeding, mice were sacrificed and lesion size and collagen content was determined. Lesion size (B) or collagen content (C) was not affected by adaptively transferring CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells.

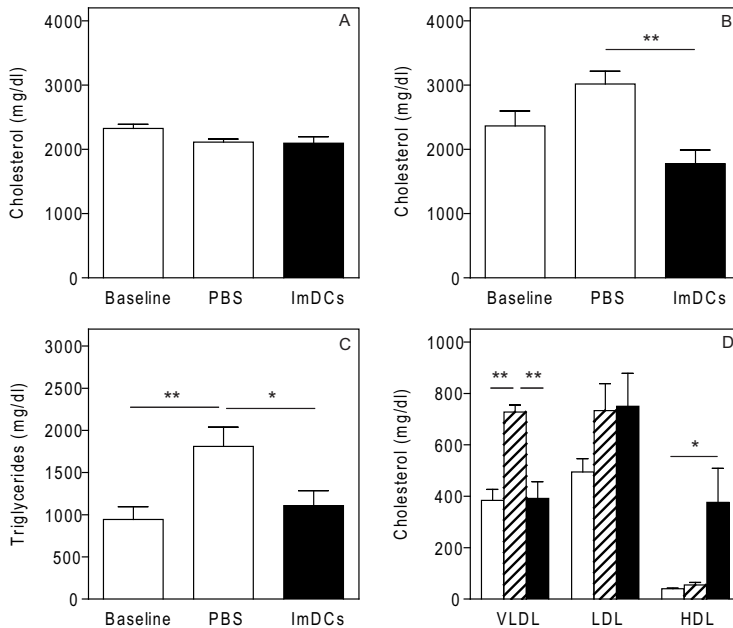
**Treatment with ImDCs affects cholesterol metabolism**

Both body weight and total cholesterol levels were determined at different time points during the experiment. Body weight did not vary between groups throughout the experiment. After 20 weeks of diet, mice were equally distributed according to their cholesterol levels prior to injection of the ImDCs (Figure 10A). Cholesterol levels in the mice treated with ImDCs showed a trend towards a reduction in cholesterol levels already three weeks after injection ( $P=0.08$ ) (Figure 9B). At 10 weeks after treatment with ImDCs, a 41% reduction in plasma cholesterol level compared to the PBS treated mice was observed ( $P=0.0018$ ; Figure 10B), while triglyceride levels were also lowered ( $P=0.0079$ ; Figure 10C). In addition, mice treated with ImDCs presented a more beneficial cholesterol profile, with decreased VLDL levels and increased HDL levels (Figure 10D). When determining the expression levels of genes involved in lipid metabolism, we observed an initial increase in relative expression of hepatic lipase, lipoprotein lipase, ABCA1 and ABCG1 three days after the last injection in mice injected with ImDCs (Figure 9A), but these expression levels were normalized three weeks after injection (data not shown). However when we assessed gene expression levels at ten weeks after injection, we observed a lowered expression of LIGHT and Lymphotoxin-α (Figure 11).



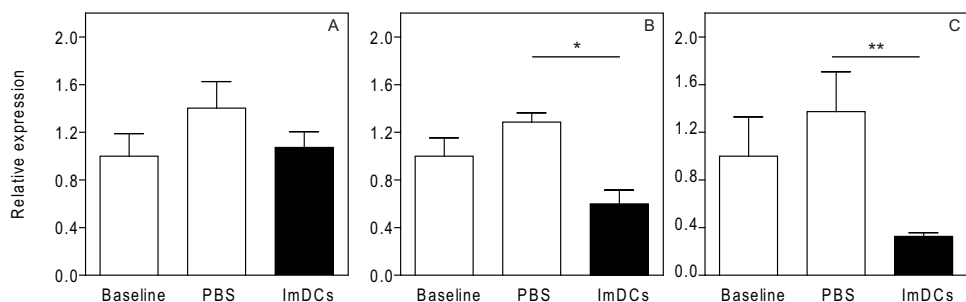
**Figure 9: Short-term effect of ImDCs treatment on expression of genes involved in lipid metabolism in liver**

Mice were given 3 injections of ImDCs and sacrificed after 3 days. mRNA was isolated from liver and the expression of hepatic lipase, lipoprotein lipase, ABCA1 and ABCG1 was determined relative to the average expression of household genes (18s, 36B4,  $\beta$ -actin and GAPDH) (A). Expression of genes from mice treated with PBS was set to 1. (B) Cholesterol levels were determined 3 days and 3 weeks after injection of ImDCs (B) (\* $P$ <0.05).



**Figure 10: Treatment with ImDCs lowers serum cholesterol and triglyceride levels**

Cholesterol levels in plasma were determined after 20 weeks of diet feeding to distribute the mice equally between groups before the injection of ImDCs or the sacrifice of the baseline group (A). At sacrifice, mice treated with ImDCs have lowered cholesterol levels (B) and triglyceride levels (C). Serum was separated on a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles. The area under the curve was calculated. Mice treated with ImDCs had a more beneficial lipoprotein profile (D) (\* $P$ <0.05; \*\* $P$ <0.01).



**Figure 11: Expression of LIGHT and Lymphotoxin- $\alpha$  is reduced in mice treated with ImDCs**

Liver mRNA from LDLR<sup>-/-</sup> mice with established lesions was isolated 10 weeks after injections. The expression of LT $\beta$  receptor (A), LIGHT (B) and LT- $\alpha$  (C) was determined relative to the average expression of household genes (18s, 36B4,  $\beta$ -actin and GAPDH). Expression of genes from mice treated with PBS was set to 1 (\*= $P$ <0.05; \*\*= $P$ <0.01).

## Discussion

As the importance of inflammatory and immune responses in atherosclerosis becomes increasingly clear, it is fascinating to exploit this information in order to beneficially influence the outcome of atherosclerosis by immunotherapy. Especially dendritic cells (DCs) form an interesting target for immunotherapy. The therapeutic potential of immature DCs (ImDCs) has been shown in animal models for autoimmune diseases such as collagen-induced arthritis<sup>13, 16</sup>, experimental autoimmune encephalomyelitis<sup>17</sup>, and type 1 diabetes.<sup>18</sup> Dyslipidemia may impair the migration of antigen loaded DCs in the periphery to the lymph nodes.<sup>19</sup> In addition with the assumption that continuous presentation of self-antigens from the atherosclerotic lesions by DCs in the lymph nodes is essential for tolerance induction and maintenance, it may be hypothesized that the entrapment of DCs in peripheral tissues will play a major role in breaking tolerance and in the induction of inflammatory responses. Moreover, the maturation status of DCs will determine whether DCs will induce immunity or tolerance.<sup>20</sup> We hypothesized that the injection of ImDCs may overcome the impaired migration towards the lymph nodes and may reestablish tolerance towards atherosclerosis-related antigens. Immature DCs or semi-matured DCs will induce tolerance, whereas, mature DCs are fully functional to activate T cells.<sup>21</sup> *Krupa et al.* elegantly showed that in granulomatous arteritis DCs are trapped in the inflamed adventitia and are of a mature phenotype. A subset of these DCs expresses CD86, which is mandatory to stimulate T cells.<sup>22</sup> Therefore trapped DCs will play a major role in sustaining the inflammatory status of the atherosclerotic plaque. In the present study we show for the first time that ImDC-treatment prevents progression of plaque growth in a mouse model of established atherosclerosis. Lesion progression was stopped at all major sites of plaque development. To elude the mechanism, we intensively assessed the effect of ImDCs treatment on the cellular response after 3 days, 3 weeks and 10 weeks after injection. We observed an increase in the number of tolerogenic CD4<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup> cells in liver and spleen in mice that received ImDCs as soon as three days after injection.

These CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells have been shown to have a protective activity in animal models of auto-immune diseases.<sup>13-15, 23</sup> Therefore, we isolated purified CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells from LDLr<sup>-/-</sup> mice after ImDCs treatment and adaptively transferred them into recipient LDLr<sup>-/-</sup> with established lesions. Unexpectedly, the transfer of the CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells did however not affect the progression of atherosclerosis. Neither the transfer of the CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>+</sup> cells, nor the transfer of CD4<sup>+</sup>TCRβ<sup>+</sup>DX5<sup>-</sup> cells did inhibit the progression of atherosclerosis, indicating that the effect of ImDCs treatment on atherosclerosis progression results from other effects than the induction of the immunoregulatory DX5 cells. Another important potential tolerogenic feature of ImDCs is their ability to induce regulatory T cells.<sup>24</sup> Puijvelde *et al.* showed that oral tolerance to oxLDL and HSP60 lead to an increase of Tregs and a subsequent reduction in lesion size.<sup>11, 25</sup> In accordance, Ait-Oufella *et al.* showed that depletion of Tregs with anti-CD25 treatment aggravated atherosclerosis in ApoE<sup>-/-</sup> mice.<sup>12, 26</sup> Taken together, these data imply an important protective role of Tregs in atherosclerosis. Also in our present study we observed that treatment with ImDCs induced Tregs in the circulation three weeks after injection which was continued until 10 weeks after treatment. This increase of Tregs could indicate a reduced immune activation after ImDCs treatment. Another frequently used measurement of immune activation is the CD4/CD8 ratio because it presents the ratio between CD4<sup>+</sup> T helper cells to cytotoxic/suppressor CD8<sup>+</sup> T cells. A decreased CD4/CD8 ratio is therefore an indication of tolerance and an increased intrasplenic/intrahepatic ratio indicates tolerance induced trapping of CD8<sup>+</sup> cells in the liver.<sup>27, 28</sup> Indeed we observed that the peripheral/intrahepatic ratio increased shortly after ImDCs injection. Overall, these data suggest that treatment with ImDCs reduced the immune activation as indicated by increased numbers of Tregs in the circulation and a decreased CD4/CD8 ratio. In addition we observed in the present study that the injection of ImDCs also affected cholesterol metabolism. The cholesterol lowering capacity of DCs has recently been described in a recent study by *Gautier et al.* who show that plasma cholesterol levels are lowered after transferring bone-marrow from transgenic mice expressing human Bcl-2 under the murine CD11c promoter into LDLr<sup>-/-</sup> and ApoE<sup>-/-</sup> mice. DCs from these mice have enhanced resistance to apoptosis which results in enhanced lifespan of DCs. In addition, diphtheria-toxin induced CD11c<sup>+</sup> cell depletion increased cholesterol levels but lesion size was not affected.<sup>29</sup> In contrast, *Stoneman et al.* showed that cholesterol levels were not affected after diphtheria-toxin induced CD11b<sup>+</sup> macrophage depletion, indicating that the cholesterol lowering capacity may be restricted to the function of DCs.<sup>30</sup> We now provide more detailed information as to how DCs influence the cholesterol metabolism. As early as three days after injection, the expression of ABCA1, ABCG1, hepatic lipase and lipoprotein lipase mRNA was increased. These genes play an important role in cholesterol transport and metabolism. Although, the expression level of these genes was normalized three weeks after injection, plasma cholesterol levels were already decreased. This decrease in cholesterol continued during the course of the experiment and after ten weeks, mice treated with ImDCs displayed lowered cholesterol and triglyceride levels and a more beneficial lipoprotein profile with decreased VLDL and increased HDL levels, which may contribute to the beneficial effect of ImDCs. It remains however unclear how exactly ImDCs injection affects



cholesterol metabolism. More research on gene expression of a more expanded cohort of genes involved in cholesterol metabolism in time is therefore needed. However, at present we observed a lower expression of LIGHT and lymphotoxin- $\alpha$  ten weeks after treatment. This decrease can also be correlated to the lowered cholesterol and triglyceride levels observed. *Lo et al.* described that LIGHT-overexpressing mice displayed hypercholesterolemia and hypertriglyceridemia even on a chow diet.<sup>31</sup> In another study it was shown that loss of Lymphotoxin- $\alpha$  (LT- $\alpha$ ) led to reduced cholesterol levels but increased HDL in LT- $\alpha$  KO mice and a subsequent reduction of atherosclerosis in ApoE<sup>-/-</sup> mice.<sup>32</sup> Also in a clinical setting there is evidence that blockage of LIGHT and TNF has a beneficial effect on the lipoprotein profile. In a recent study lipoprotein profiles of patients treated with infliximab or etanercept were compared. The blockade of LT- $\alpha$  is a feature of etanercept. Higher HDL levels were observed in patients treated with etanercept, while patients treated with infliximab showed increased LDL levels and total cholesterol after one year of treatment.<sup>33</sup> In addition, rheumatoid arthritis patients have an improved atherogenic index after anti-TNF treatment with adalimumab.<sup>34</sup> Together these data support our findings of lowered cholesterol and a more beneficial lipoprotein profile and that this decrease can be correlated to the lowering of LIGHT and lymphotoxin- $\alpha$ . *Laxton et al.* showed that there is a strong association between susceptibility for myocardial infarction and a polymorphism in the Lymphotoxin- $\alpha$  gene.<sup>35</sup> More importantly, targeted depletion of LT- $\alpha$  expressing Th1 and Th17 cells inhibits autoimmune disease.<sup>36</sup> Therefore, reduced levels of LIGHT and lymphotoxin- $\alpha$  may indicate a reduced inflammatory T cell phenotype in our ImDCs treated mice. In conclusion, we show here for the first time that treatment with ImDCs impairs plaque growth in a mouse model for established atherosclerosis by inducing tolerance and affecting cholesterol metabolism. Therefore, our data provide strong evidence for the therapeutic potential of dendritic cells in a clinically relevant setting. In addition, the use of DCs in clinical trials has now provided proof of principle and has shown that the technique is safe with minimal side-effects, reviewed by *Tacke et al.*<sup>37</sup> Therefore, the use of DCs to shift immune responses towards an anti-atherogenic outcome could also prove to be beneficial in the treatment of patients with cardiovascular disease.

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