Regulation of immune responses in atherosclerosis

Foks, A.C.

Chapter 7

Vaccination against Foxp3^{+} regulatory T cells aggravates atherosclerosis

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Abstract

Objective: Regulatory T cells are crucial for immune homeostasis and an impaired regulatory T cell function results in many pathological conditions. Regulatory T cells have already been described to be protective in atherosclerosis. However the exact contribution of Foxp3 expressing natural regulatory T cells in atherosclerosis has not been elucidated yet.

Methods and Results: In this study we vaccinated LDL receptor deficient mice with dendritic cells, which are transfected with Foxp3 encoding mRNA, and studied the effect on initial atherosclerosis. Vaccination against Foxp3 resulted in a reduction of $Foxp3^+$ regulatory T cells in several organs and in an increase in initial atherosclerotic lesion formation. Furthermore we observed an increase in plaque cellularity and increased T cell proliferation in the Foxp3 vaccinated mice.

Conclusions: We further establish the protective role of Tregs in atherosclerosis. The results illustrate the important role for Foxp3 expressing regulatory T cells in atherosclerosis, thereby providing a potential opportunity for therapeutic intervention against this disease.

Introduction

Atherosclerosis is an autoimmune like disease, in which both innate and adaptive immune responses are involved.[1] T helper (Th) cells are crucial for an adequate immune response and can be divided in Th1 and Th2 cells. Several studies show that inflammatory processes in atherosclerosis are associated with a Th1-driven immune response (IFN-γ, IL-12), while the Th2 cells (IL-5 and IL-13) exert an anti-atherogenic role.[2, 3] It was postulated that an imbalance between Th1 and Th2 cells was, at least partially, responsible for the development of atherosclerotic lesions. However, more recently, IL-4, a Th2-cytokine, was found to be pro-atherogenic in early lesion formation.[4, 5] This finding, together with studies establishing an anti-atherogenic role for regulatory T cells (Tregs), suggested another mechanism of immune regulation in atherosclerosis, where T cells (both Th1 and Th2) are suppressed by regulatory T cells (Tregs).

Tregs are characterized by the expression of both CD4 and CD25 and are subdivided in adaptive Tregs and natural Tregs. Adaptive Tregs develop from naive T cells in the periphery and can produce IL-10 (Tr1 cells) and TGF-β (Th3 cells). Natural Tregs originate from the thymus as $CD4^+CD25^+$ cells and exert their suppressive function especially via cell-cell contact and membrane bound TGF-β and CTLA-4. Forkhead box protein P3 (Foxp3) is characteristically expressed in this subclass of Tregs and this transcription factor is necessary for the development of Tregs. Deficiency in Foxp3 leads to a lack of Tregs and severe auto-immune disorders.[6-9]

Recently, we showed that oral administration of atherosclerosis-related antigens (HSP60 and oxLDL) increases the number of Foxp3-expressing Tregs in several organs, which leads to a decrease in development of atherosclerotic lesions in $LDLr^{-/-}$ mice.[10, 11] These results are in line with studies on the role of Tregs in atherosclerosis after oral and nasal tolerance induction[12-14] but our studies specifically demonstrate the contribution of $Foxp3^+$ Tregs. Furthermore, a study by Mallat and colleagues showed that a transfer of Tregs reduced lesion formation in $ApoE^{-/-}$ mice[15], while others showed that treatment of $ApoE^{-/-}$ mice with a depleting CD25-specific antibody (PC61), results in an increase in lesion size.[16] Additionally, bone marrow transplantation of $CD80^{-/-}$ $CD86^{-/-}$ bone marrow into $LDLr^{-/-}$ mice results in a decrease in the number of Tregs and an increase in lesion size, again indicating an inverse relationship between the presence of Tregs and atherosclerotic lesion development.[16]

However, these studies do not directly demonstrate the role of Foxp3 expressing cells in atherosclerosis. To specifically establish the role of Foxp3-expressing Tregs, we targeted Foxp3 expressing cells using DCs electroporated with mRNA encoding for Foxp3 as described by Nair et al., which induces a cytotoxic T lymphocyte response against Foxp3.[17] In the present study, we show that vaccination against Foxp3 results in a reduction of $Foxp3^+$ Tregs and a subsequent increase in initial atherosclerotic lesion formation, thereby establishing a prominent role for $Foxp3^+$ Tregs in this process.

Material 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. Female LDLr$^{-/-}$ mice were obtained from Jackson Laboratories. Male C57BL/6J mice were from Charles River Laboratories. All 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). All mice used were 10-12 weeks of age. Diet and water were administered *ad libitum*.

Synthesis of Foxp3 and GFP mRNA

The pSP73-Spf/Foxp3/A64 construct was kindly provided by E. Gilboa (Duke University Medical Center, Durham, NC, USA).[17] As a control we created a pSP73-Spf/eGFP/A64 construct. The pSP73-Spf/Foxp3/A64 and pSP73-Spf/eGFP/A64 constructs were used as a DNA template in a T7 mMessage mMachine® (Ambion, Austin, TX) reaction to produce large amounts of capped Foxp3 and GFP *in vitro* transcribed mRNA. To eliminate excessive DNA, a TurboDNase® (Ambion, Austin, TX) treatment was performed. The Megaclear Kit® (Ambion, Austin, TX) was used for purifying mRNA from the *in vitro* transcription reactions. All reactions were performed according to the manufacturer's protocol.

Generation and assessment of the DC based vaccine

Bone marrow cells were isolated from the tibia and femora of C57BL/6J mice. Cells were pooled and cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) for 10 days in IMDM supplemented with 8% fetal calf serum (FCS, PAA), 100 U/mL streptomycin/penicillin (PAA), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μM β-mercaptoethanol. Hereafter, the DCs were harvested, washed, and resuspended in Opti-MEM (GIBCO, Grand Island, NY). The used DC culture medium was saved as conditioned media for later use. 5 x 10^6 DCs in 200 μl Opti-MEM were electroporated with either GFP or Foxp3 mRNA as described by Nair et al.[18] After electroporation the DCs were transferred to culture petridishes containing GM-CSF and a 1:1 combination of conditioned DC growth medium and fresh medium. Transfected DCs were incubated at 37°C, 5% CO_2 overnight, and subsequently were washed 2 times in PBS before vaccination. As a control for maturation of DCs, LPS (1 μg/mL) was added to one group of DCs for 24 hours. To assess the maturation profile, the electroporated DCs were stained against surface markers (CD80-FITC, CD86-PE and CD40-PE) and analyzed using FACS. To detect intracellular Foxp3 expression in the electroporated DCs, the cells were stained with Foxp3-APC. For intracellular staining of Foxp3, DCs were fixated and permeabilized overnight and subsequent stained against Foxp3. All antibodies were purchased from eBioscience (Belgium) and used for FACS analysis according the manufacturer's protocol. Total RNA was isolated from the electroporated DCs using the GTC method.[19] cDNA synthesis was performed using Revert

Aid™ M-MuZV Reverse Transcriptase (Fermentas Life Science). Quantitative Foxp3 gene expression (5'-GGAGCCGCAAGCTAAAAGC-3' and 5'-TGCCTTCGTGCCCACTGT-3') analysis was performed on a 7500 fast Real-Time PCR System (Applied Biosystem) using SYBR Green technology. Acidic ribosomal phosphoprotein PO (36B4; 5'-GGACCCGAGAAGACCTCCTT-3' and 5'-GCACATCACTCAGAATTTCAATGG-3') and hypoxanthine phosphoribosyl transferase (HPRT; 5'-TTGCTCGAGATGTCATGAAGGA-3' and 5'-AGCAGGTCAGCAAAGAACTTATAG-3') were used as reference genes.

Vaccination and the induction of atherosclerosis

Mice were injected with $5x10^5$ DCs in 100 µl per mouse (GFP n=14, Foxp3 n=13) subcutaneously at the base of the ear pinna at day 0. Mice of the control group (n=15) were injected with 100 µl PBS. After treatment, the mice were fed a Western-type diet (0.25% cholesterol and 15% cocoa butter) for 8 weeks to induce hypercholesterolemia and atherosclerosis. At sacrifice, tissues were harvested after *in situ* perfusion using PBS and subsequent perfusion with Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Tissues were snap-frozen in nitrogen and stored at –80 °C until further use. During the experiment blood samples were obtained by tail vein bleeding at various time points. The concentration of serum cholesterol was determined using an enzymatic colorimetric procedure (Roche/Hitachi). Precipath (Roche/Hitachi, Mannheim, Germany) was used as an internal standard. Blood samples of week 5 were also used to determine the percentage of Tregs in blood using flow cytometry as described below.

Flow cytometry

At sacrifice, Peripheral Blood Mononuclear Cells (PBMCs) were isolated via orbital bleeding and erythrocytes were removed by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Spleens, heart lymph nodes (HLN) and mesenteric lymph nodes (MLN) were dissected from the mice (n=5/group) and a single cell suspension was obtained by passing the organs through a 70 µm cell strainer (Falcon, The Netherlands; n=5/group). Cells were stained with surface markers (0.25 µg antibody/300.000 cells) and subsequently analyzed by flow cytometric analysis. For the detection of $CD4^+CD25^+Foxp3^+$ T cells, the spleen, blood, MLN and HLN were stained with CD4-FITC and CD25-PE and subsequently intracellularly with Foxp3-APC. All antibodies were purchased from eBioscience (Immunosource, Belgium). All data were acquired on a FACSCalibur (Becton Dickinson, Mountain View, CA) and analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Spleen cell proliferation

Splenocytes (n=5/group) were cultured for 72 hours in triplicate in a 96-wells round-bottom plate (2×10^5 cells/well, Greiner Bio-One) in RPMI 1640 supplemented with L-Glutamine, 10% FCS and 100 U/mL streptomycin/penicillin. As a positive control cells were stimulated with Concanavalin A (2 µg/mL, Con A, Sigma Diagnostics, MO).

Proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well, Amersham Biosciences, The Netherlands) for the last 16 hours. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The proliferation is expressed in disintegrations per minute (dpm).

Histological analysis

The heart was embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and cryosections (10 µm) of the aortic root containing the three aortic valves were made. Cryosections were routinely stained with Oil-Red-O and hematoxylin (Sigma Diagnostics, MO). Corresponding sections on separate slides were also stained for collagen, macrophages and fibroblasts using Masson's Trichrome staining (Sigma Diagnostics), MoMa-2 antibody (Research Diagnostics Inc.), and ER-TR7 antibody (AbD Serotec), respectively, according to manufacturer's protocols. For the cellularity assessment a hematoxylin staining was performed. The different histological stainings were quantified using a Leica DM-RE microscope and Leica Qwin Imaging software (Leica Ltd., Germany).

Statistical Analysis

All data are expressed as mean±SEM. The two-tailed student's T-test was used to compare individual groups of mice or cells. When indicated, a Mann-Whitney test was used to analyze not normally distributed data. The frequency of thickened cap structure was analyzed by a Fisher's exact test. P values of <0.05 were considered significant.

Results

A DC based vaccination strategy against Foxp3

To test whether a reduction in Foxp3-expressing Tregs affects atherosclerosis, a DC

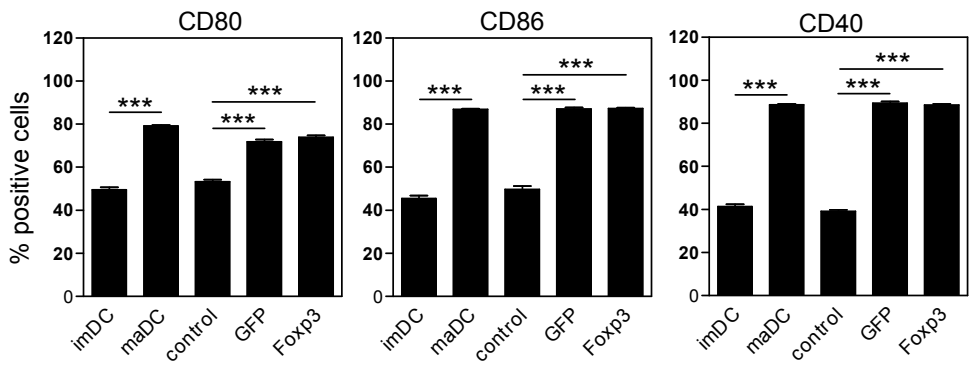


Figure 1. Expression of costimulatory molecules on electroporated DCs. After electroporation with either PBS (control, n=3), GFP mRNA (n=3) or Foxp3 mRNA (n=3) the DCs were cultured o/n. One group of DCs were stimulated with LPS as a positive control for maturation (mDCs). Unstimulated DCs (imDCs) were used as a negative control for maturation. DCs were subsequently stained for CD80, CD86 and CD40 and analyzed by FACS. ***$P<0.001$

based vaccination strategy was used in which DCs were transfected with mRNA encoding for Foxp3. A significant increase in the surface expression of the co-stimulatory molecules, CD80, CD86 and CD40 was observed in DCs that were electroporated with mRNA encoding Foxp3 or GFP, when compared with electroporation without mRNA (Control) and immature DCs (imDCs). The increase is comparable to the LPS induced maturation of DCs (mDCs) (Figure 1). After electroporation with mRNA encoding for Foxp3 a strong increase in Foxp3 expression in DCs was observed on mRNA level, which indicated a successful transfection of the DCs (Figure 2A). To determine the intracellular protein expression of Foxp3, an intracellular Foxp3 FACS staining was performed (Figure 2B and C). Foxp3 transfected DCs expressed 6.3-fold more intracellular Foxp3 compared to GFP transfected DC, which expressed no Foxp3.

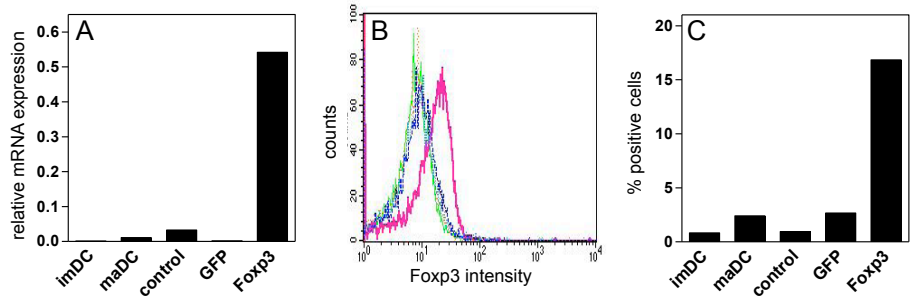


Figure 2. Assessment of Foxp3 specific mRNA and the subsequent expression in DCs. After electroporation of DCs with mRNA encoding for Foxp3 or GFP, total RNA was isolated and cDNA was synthesized. Subsequently the amount of mRNA was detected with Foxp3 specific qPCR primers (A). The expression is relative to HPRT and 36B4. Next we determined, with FACS analysis, the intracellular expression of Foxp3 in PBS (green), GFP (blue) and Foxp3 (pink) electroporated DCs. Additionally, electroporated DCs were cultured o/n and stained intracellularly for Foxp3 and analyzed by FACS. A representative histogram (B) and percentage of intracellular Foxp3 expression is depicted (C).

Vaccination against Foxp3 reduces the number of Tregs

To asses the efficacy of the Foxp3 vaccine, LDLr$^{-/-}$ mice were vaccinated with Foxp3 electroporated DCs to induce a cytotoxic immune response against Foxp3 as described by Nair et al.[17] As a control, mice were vaccinated with DCs electroporated with mRNA encoding for GFP. Before vaccination, the number of Tregs in blood in all groups of mice was equal, but five weeks after vaccination a 34% reduction in the number of Foxp3$^+$ Tregs in Foxp3 vaccinated mice was observed, compared to GFP vaccinated mice (Figure 3A; 0.773±0.032% versus 1.175±0.065%; P<0.001). Upon sacrifice, vaccination with Foxp3 transfected DCs (black bars; Figure 3B) resulted in a 27-30% decrease in Foxp3$^+$ Tregs in blood (0.67±0.045% versus 0.92±0.096%, P<0.05), spleen (2.88±0.24% versus 3.94±0.34%, P<0.05), MLN (3.59±0.10% versus 5.01±0.45%, P<0.05) and HLN (3.68±0.08% versus 5.50±0.46%, P<0.01), when compared to mice vaccinated with DCs electroporated with mRNA encoding for GFP (grey bars; Figure 3B). As a control for the DC vaccination, one group of mice was treated with PBS. We observed no differences in the numbers of Foxp3$^+$ Tregs in lymphoid organs and blood between mice treated with PBS and mice vaccinated with

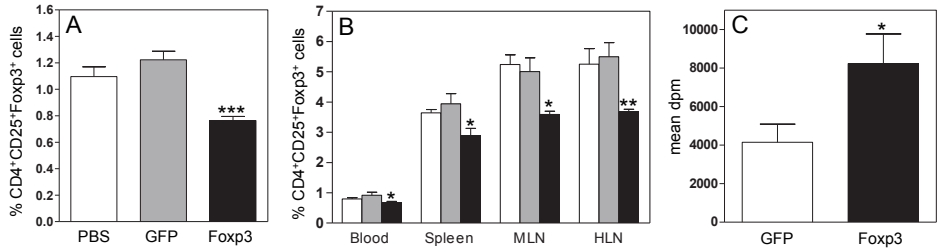


Figure 3. Effect of vaccination against Foxp3 on the percentage of Tregs in lymphoid organs and on spleen cell proliferation. LDLr$^{-/-}$ mice were vaccinated against GFP or Foxp3 using electroporated DCs. As a second control, one group of mice was vaccinated with PBS. Five weeks after vaccination blood was taken from the mice vaccinated with either PBS (A, n=5, white bar), GFP (n=5, grey bar) or Foxp3 (n=5, black bar) and was analyzed for CD4$^+$CD25$^+$Foxp3$^+$ cells using FACS analysis. Eight weeks after vaccination the mice vaccinated with either PBS (B, n=5, white bars), GFP (n=5, grey bars) or Foxp3 (n=5, black bars) were sacrificed. Blood, spleen, MLN and HLN, were isolated and the number of CD4$^+$CD25$^+$Foxp3$^+$ cells in these organs was determined. The effect of vaccination against Foxp3 on spleen cell proliferation was determined by culturing splenocytes isolated after 8 weeks *ex vivo* (C). Proliferation was assessed by the amount of ^{3}H-thymidine incorporation in dividing cells. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The proliferation is expressed in disintegrations per minute (dpm). *P<0.05, **P<0.01, ***P<0.001

DCs electroporated with mRNA encoding for GFP (white bars; Figure 3A and B). Regulatory T cells have an inhibitory effect on T cell proliferation. Therefore we investigated whether a vaccination against Foxp3 and a subsequent reduction in the number of Tregs influences the proliferation of splenic T cells. We observed a significant 1.9-fold increase in spleen cell proliferation compared to the GFP vaccinated mice (Figure 3C: 4148.5±941.4 dpm versus 8230.5±1542.5 dpm P<0.05).

Vaccination against Foxp3 increased lesion formation in LDLr$^{-/-}$ mice

Eight weeks after vaccination and Western-type diet feeding the plaque size at the aortic root was analyzed. Representative slides of the aortic root of GFP and Foxp3 vaccinated mice are shown in Figure 4A and B, respectively. Mice vaccinated against Foxp3 showed a significant 34% increase in plaque size compared to the GFP vaccinated mice (Figure 4C; 538.932±46.043 μm^2 versus 382.865±29.044 μm^2,

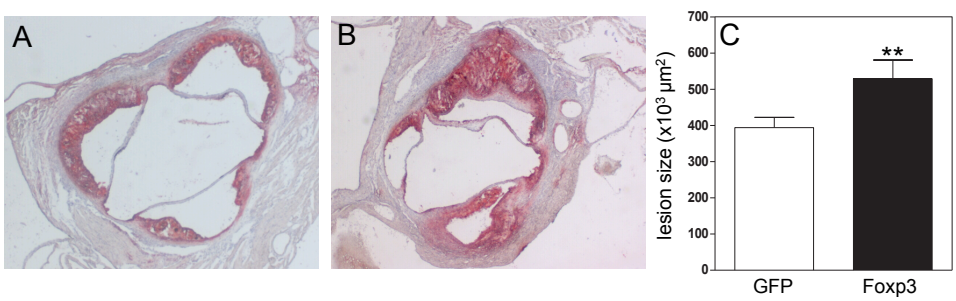


Figure 4. Effect of vaccination against Foxp3 on initial atherosclerotic lesion development. Eight weeks after vaccination and Western-type diet feeding the LDLr$^{-/-}$ mice were sacrificed and the hearts of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with Oil-Red-O and hematoxylin. The lesions were quantified and the plaque size was determined (C). **P<0.01

P<0.01). During the experiment, all mice developed hypercholesterolemia, however no significant differences in serum cholesterol levels and body weight (data not shown) were observed between the different groups of mice. No differences in plaque size were observed between PBS treated mice and mice vaccinated against GFP (data not shown).

Vaccination against Foxp3 results in increased plaque cellularity

To investigate whether there is a change in plaque composition in the enlarged initial plaques of the Foxp3 vaccinated mice the number of cells within the lesions were quantified. The plaques of Foxp3 vaccinated mice showed a 27% increase in cellularity compared to GFP vaccinated mice (Figure 5A-C; $3.84x10^{-3}\pm0.19x10^{-3}$ cells/μm^2 versus $2.81x10^{-3}\pm0.79x10^{-3}$ cells/μm^2, respectively; *P*<0.01). Subsequently we determined the plaque stability by measuring both the amount of collagen within the plaque and the fibrous cap thickness. The collagen content of the plaque was not significantly changed between the Foxp3 and GFP vaccinated mice (Figure 5D-F, 9.0±1.1% versus 6.5±1.9%, respectively).

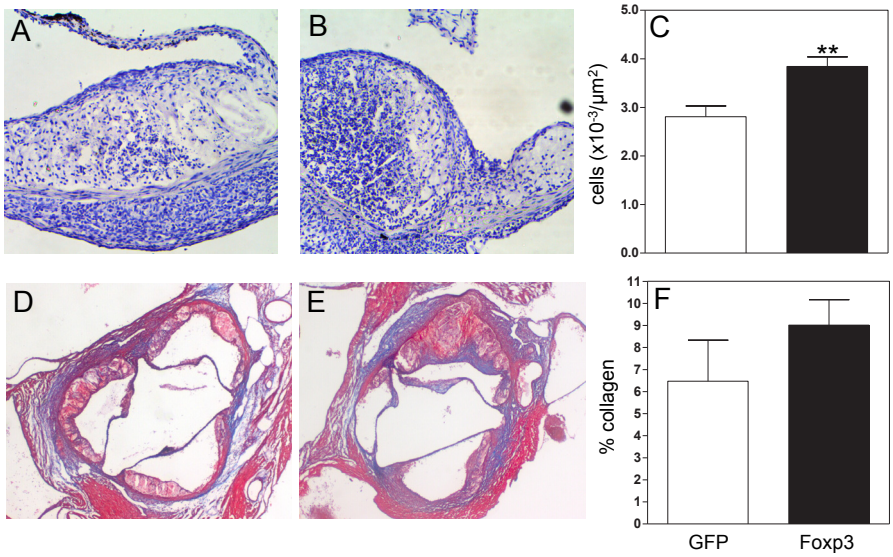
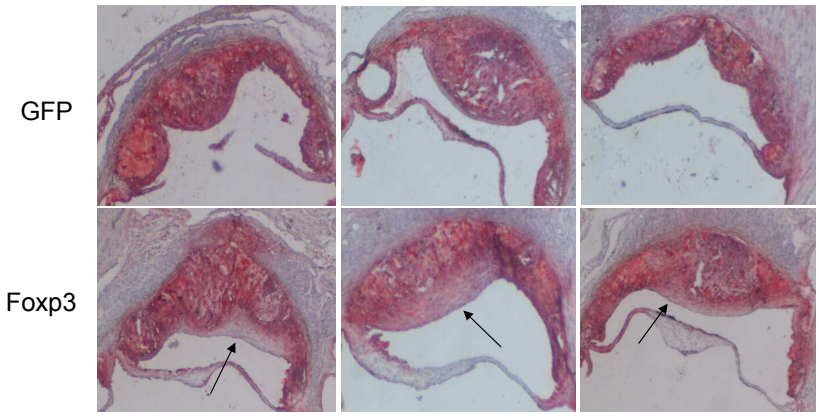


Figure 5. Effect of vaccination against Foxp3 on plaque composition. Eight weeks after vaccination and Western-type diet feeding the $LDLr^{-/-}$ mice were sacrificed and the hearts of GFP treated (A) and Foxp3 treated (B) mice were sectioned and stained with hematoxylin to stain the nucleus of the cells within the lesion. The number of nuclei was quantified (C). Sections of the hearts of GFP treated (D) and Foxp3 treated (E) mice were also analyzed for collagen content using the Masson's Trichrome staining which stains collagen blue. The percentage of collagen relative to the lesion size was determined (F). ***P*<0.01

Additionally, we noticed that the lesions in 9 of the 13 Foxp3-vaccinated mice displayed significant characteristic changes in morphology. These lesions are characterized by a thickened fibrous cap-like structure (Figure 6, arrows). This cap however does not cover the entire plaque and seems to grow into the lesion. In addition, this cap-like

structure does not seem to provide a stable phenotype. When compared with GFP vaccinated mice in which 2 out of 14 mice showed these thickened structures, a significant difference is observed when a Fisher's exact test is performed (Figure 6, $P<0.01$). These thickened structures do not contain any macrophages or fibroblasts and overall there was no significant difference in cap thickness in the different groups.



	No thickened cap	Thickened cap
GFP	12	2
Foxp3	4	9

Figure 6. Thickened cap structure in Foxp3 vaccinated mice. Within the Foxp3 vaccinated group more thickened structures in the fibrous cap (arrows) were observed when compared to the GFP vaccinated mice. Frequency of thickened structure between GFP and Foxp3 vaccinated mice is depicted in the table. A Fisher's exact test was used ($P<0.01$).

Discussion

In this study we demonstrate that vaccination against Foxp3$^+$ Tregs aggravates atherosclerotic lesion formation, thereby directly establishing the protective role of Foxp3$^+$ Tregs in atherosclerosis. The beneficial role of Tregs in atherosclerosis is already indicated in a number of publications.[16, 20] We showed before that induction of Foxp3 positive Tregs via oral tolerance induction against HSP60 and oxLDL reduces atherosclerotic lesion formation.[10, 11] Furthermore, Ait-Oufella and colleagues demonstrated that a deficiency in costimulatory molecules promotes atherosclerosis because of a decrease in the number of Tregs and showed that a depletion of CD25$^+$ cells using anti-CD25 antibodies increased lesion formation.[16] However, the final proof for the involvement of Foxp3 expressing T cells in atherosclerosis has not been provided since CD25 is not exclusively expressed on Tregs, as it is also present on activated T cells[21], NK cells[22] and myeloid DCs.[23]

To specifically study the role of Foxp3 positive Tregs in atherosclerosis, we vaccinated atherosclerosis prone mice against Foxp3 using a DC vaccination strategy. DCs are

electroporated with mRNA encoding for Foxp3 and this approach, described by Nair et al, results in a cytotoxic T lymphocyte (CTL) response against Foxp3 and a subsequent depletion of Foxp3+ Tregs.[17] As a control, mRNA encoding for GFP was used to exclude that mRNA electroporation into DCs activates the DCs leading to immune modulatory effects. In all the experiments, the treatment with GFP electroporated DCs was compared with PBS treatment and no differences were observed between both control treatments. After electroporation of DCs with mRNA, either with GFP or with Foxp3, an activated phenotype of DCs was observed. This was not observed when DCs were electroporated without mRNA. It is known that single stranded RNA (ssRNA), such as mRNA, is a natural ligand for Toll like receptors 7 and 8,[24, 25] which may explain the activated phenotype of the DCs after electroporation with mRNA.

We successfully demonstrated that the DCs were transfected with the mRNA coding for Foxp3 via qPCR and FACS analysis. Foxp3 is a nuclear product and is not expressed on the cell surface. However, we use a truncated Foxp3 in which the nuclear localization sequence is removed. This results in relatively high concentration of cytosolic Foxp3 protein and therefore may result in the cross-presentation of Foxp3-peptides on MHC class I. Furthermore, mRNA may act as a natural agonist of TLR7/8 and thereby even further enhance cross-presentation, which is already described by other groups.[24-27] This may lead to the presentation of Foxp3 peptides to CD8+ T cells, which subsequently target Foxp3 expressing cells.

Our present data show a significant reduction in Foxp3+ Tregs in blood 5 weeks after vaccination. A similar reduction in Foxp3+ Tregs was observed within the blood, HLN, MLN and spleen, 8 weeks after vaccination, indicating a systemic reduction in Foxp3+ Tregs and a persistent effect of the vaccination. As shown before by Nair et al.[17] this reduction in Tregs is caused by the induction of a robust Foxp3-specific cytotoxic T lymphocyte response. Additionally, we observed an increased spleen cell proliferation in the Foxp3 vaccinated mice compared with GFP vaccinated mice which is indicative for a reduced number of Tregs since these cells suppress effector T cells. The reduction in Foxp3+ Tregs resulted in a 34% increase in initial lesion size. The increase in atherosclerosis is not related to a change in total cholesterol serum levels since there is no significant difference in cholesterol levels between the different groups.

Besides an increase in lesion size, vaccination against Foxp3 also induced a 30% increase in cellularity of the initial lesions. The increased cellularity may indicate an increase in inflammation within the lesion. This may be caused by an increase in proliferation of inflammatory cells or by an increase in influx of inflammatory cells, which in normal conditions would be under the control of Tregs. The increase in cellularity is not caused by an accumulation of macrophages or fibroblasts.

In addition to plaque cellularity, we analyzed the plaque stability but no differences in both the collagen content and the fibrous cap thickness were observed between GFP and Foxp3 vaccinated mice. In spite of the fact that the fibrous cap thickness was not changed, we observed significant more thickened fibrous cap-like structures in the Foxp3 vaccinated mice. These thickened structures, which also seem to grow into

the lesion, do not contain macrophages and fibroblasts and do not cover the entire plaque. The exact composition and function of these structures remains therefore unclear and needs further investigation, especially because these cap-like structures do not look like a stable fibrous cap.

The results in this study are in line with the observations that the induction of Tregs exerts an atheroprotective effect. The increase in lesion size is comparable with the decrease of initial lesion size upon the induction of Tregs via oral administration of oxLDL (30.0%) or HSP60 (27.5%).[10, 11] In conclusion, we confirmed the protective role of Foxp3$^+$ Tregs in atherosclerosis by vaccinating LDLr$^{-/-}$ mice using a DC based Foxp3 vaccination strategy. The results illustrate an important role for Foxp3$^+$ Tregs in atherosclerosis, thereby providing a potential opportunity for therapeutic intervention against atherosclerosis.

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