Chapter 5

Deficiency of TGF-β signaling in CD11c+ cells accelerates atherosclerosis by disrupting T-cell homeostasis

-Manuscript submitted-

D. Lievens1; K.L.L. Habets2; Y. Laouar3; A. Robertson4; L. Beckers1; E. Wijnands1; T. Rademakers5; M. van Zandvoort5; L. Boon6; M. Rudling7; R.A. Flavell8; M.J.A.P. Daemen1; J. Kuiper2; E.A. Biessen1 and E. Lutgens1,9
Abstract

TGF-β is an anti-inflammatory and pro-fibrotic cytokine that plays a bridging role between the innate and adaptive immune system. However, the exact liaison between TGF-β, the innate and the adaptive immune system in atherosclerosis is still unclear. Here we investigate the effect of deficiency of TGF-β signaling in cells of the innate immune system (ie CD11c+ DCs, NK cells and macrophages) in atherosclerosis, by using transgenic mice with a targeted functional inactivation of TGF-βRII signaling in CD11c+ cells (ApoE/-CD11cDNR). ApoE/-CD11cDNR mice were sacrificed at 20 wks of age and plaques in the aortic root were analyzed. ApoE/-CD11cDNR mice exhibited a 2-fold increase in plaque area and showed a substantial increase in CD45+ leukocytes and CD3+, CD4+ and CD8+ T-cells, while macrophage content was not affected. Moreover, a significant reduction in α-smooth muscle cell (ASMA) and collagen content was observed in plaques of ApoE/-CD11cDNR mice. FACS analysis of lymphoid organs of ApoE/-CD11cDNR mice revealed an increase in both CD4+ and CD8+ CD44^highCD62L^low effector memory T-cells, whereas the number of CD4+CD25^FoxP3^ regulatory T-cells had increased. In the supernatant of CD3/CD28 stimulated splenocytes from ApoE/-CD11cDNR mice, elevated levels of Th1 (IFN-γ), Th2 (IL-4, IL-10) and Th17 (IL-17) cytokines were detected. In conclusion, deficient TGF-β signaling in CD11c+ cells results in a disturbed T-cell homeostasis, thereby accelerating atherosclerosis and inducing a pro-inflammatory plaque phenotype.
Deficiency of TGF-β signaling in CD11c+ cells

Introduction

Atherosclerosis is a chronic inflammatory disease of the arterial wall.\textsuperscript{1-3} Immune cells from both the innate and the adaptive immune system orchestrate the formation and progression of atherosclerotic plaques by controlling their structure, composition and expansion.\textsuperscript{3} This complex interplay between different cells and cell types of the immune system is coordinated by a broad array of pro- and anti-inflammatory chemokines and cytokines. The balance between pro- and anti-inflammatory modulators is decisive in the degree of atherosclerosis progression and the plaque phenotype that develops.\textsuperscript{4-7}

One of the central regulators of the immune response is transforming growth factor beta (TGF-β).\textsuperscript{8, 9} Three highly conserved isoforms of TGF-β have been described: TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is present at high levels in healthy blood vessels and interestingly, higher levels of TGF-β1 are present in human asymptomatic lesions than in lesions from symptomatic patients.\textsuperscript{10} The closely related TGF-β2 and TGF-β3 isoforms are either absent or present at low levels.\textsuperscript{11} The classical signaling receptors for TGF-β are the TGF-β receptor I (TGF-βRI), also known as activin-like kinase (ALK), and the constitutively active type II receptor (TGF-βRII). Access of the ligands to the type I en type II receptors is regulated by soluble ligand binding proteins and by the accessory type III receptor, also known as betaglycan.\textsuperscript{12, 13} TGF-β signals in most cells via TGF-β type II receptor and ALK5.\textsuperscript{14-17}

A wide range of cell types, important in atherosclerosis, expresses TGF-β.\textsuperscript{8, 11, 18} TGF-β exerts crucial anti-inflammatory functions by inhibiting the proliferation and differentiation of T-cells and the activation of macrophages.\textsuperscript{11, 18} In endothelial cells, TGF-β inhibits the expression of adhesion molecules and chemokines required for the recruitment of leukocytes.\textsuperscript{19, 20} Moreover, TGF-β is also known to stimulate collagen production in smooth muscle cells (SMC).\textsuperscript{21, 22} In 2002 we showed that treatment of ApoE-/- mice with a soluble TGF-β-receptor II protein (TGF-βRII:Fc) that prevents TGF-β signaling, accelerated atherosclerosis. Plaques exhibited an unstable phenotype that contained low amounts of fibrosis, an increased amount of inflammatory cells, and even intraplaque hemorrhages.\textsuperscript{23} Mallat et al. who injected neutralizing antibodies against TGF-β1 into ApoE-/- mice, obtained similar results.\textsuperscript{24} Recently, Frutkin et al. showed that mice with cardiac over-expression of TGF-β reduced atherosclerosis formation in the aortic roots and prevented aortic dilatation.\textsuperscript{25} These effects could be attributed to TGF-β signaling in T-cells. Mice, with deficient TGF-β signaling in CD4+ T-cells (CD4-dnTGF-βRII), also showed accelerated plaque progression, and a shift towards an unstable atherosclerotic plaque phenotype.\textsuperscript{26} This was accompanied by an increased differentiation of T-cells towards both Th1 and Th2 phenotype. Increased levels of IFN-γ, IL-10 and IL-4 were found in cultured splenocytes.\textsuperscript{27}

Although the effects of TGF-β signaling of the adaptive immune system in atherosclerosis have been described, limited data are available on the effect of TGF-β signaling in the innate immune system. In atherosclerosis, the principal effector cells of the innate immunity are the DCs, monocytes/macrophages, neutrophils and NK cells, which all express CD11c. In the present study, we investigated the effect of TGF-β signaling in CD11c+ cells in atherosclerosis using ApoE-/- mice.
Chapter 5

that contained a dysfunctional TGF-β Receptor II (ApoE-/-CD11cDNR). We found that blockade of TGF-β signaling in CD11c accelerated atherogenesis, enhanced the influx of both CD4+ and CD8+ T-cells into the plaques and decreased fibrosis. Systemically, an increased differentiation of T-cells towards effector memory T-cells (CD44highCD62Llow) was found together with increased levels of Th1, Th2 and Th17 cytokines. Our data illustrate the important role of TGF-β as a regulator between innate and adaptive immunity in atherosclerosis.

Materials and methods

ApoE-/-CD11c-dnTGF-βRII transgenic mice

CD11c-dnTGF-βRII (CD11cDNR) mice were generated as described previously and were backcrossed to ApoE-/- mice (both genotypes were on a C57BL/6 background). In short, the human TGF-β type II receptor sequence between nucleotides -7 and +573 that encodes for the extracellular and transmembrane regions of the TGF-β type II receptor was cloned into the EcoRI site of rabbit β-globin gene exon 3 of plasmid CD11c promoter vector pDOI-5. Expression of the transgene was determined by real-time PCR in freshly isolated cell types from spleen. Samd2 phosphorylation in isolated NK cells and DCs was used as a read-out for disturbed TGF-β signaling (Supplemental Figure 1).²⁸

Supplemental Figure 1:Generation and characterization of CD11c-dnTGF-βRII transgene mice.

Transgene construct with in grey the dnTGF-βRII sequence, in white the rabbit β-globin exon gene 3 and in black the plasmid CD11c promoter (A). Normalized values to HRPT of dnTGF-βRII mRNA in cells isolated from spleen as determined by real-time PCR (B). Smad2 phosphorylation in DCs and NK cells after stimulation in the presence or absence of TGF-β with LPS or IL-12 and IL-18, respectively (C).
At the age of 20 weeks, mice were euthanized. Blood was obtained from the retro-orbital plexus and plasma cholesterol was measured using a colorimetric assay (CHOD-PAP, Roche). Spleen, liver and lymph nodes were harvested after in situ perfusion using PBS. Heart and aorta were isolated after subsequent perfusion using 1% paraformaldehyde. Hearts were frozen in Tissue-Tek (Shandon, Veldhoven, The Netherlands). Organs for autopsy were collected in 4% paraformaldehyde. All animal experiments were performed under approved Institutional Animal Care and Use Committee protocols (Yale University).

**Histology and morphometry**

Plaque area was analyzed using serial sections of 6 μm with 42 μm intervals, beginning from the onset of the aortic valves until the valves had disappeared. For histological analysis of atherosclerosis, sections were stained with hematoxylin and eosin (HE). The extent of atherosclerosis was assessed in aortic roots and plaque area was calculated by using a Leica DM3000 Light microscope (Leica Microsystems) coupled to a computerized morphometry system (Leica Qwin 3.5.1).

**Immunohistochemistry**

Corresponding sections were immunolabeled with CD45 rat monoclonal antibody (1:5000; Pharmingen) to detect all inflammatory cells, Moma-2 rat monoclonal antibody (1:50; Serotec) to detect macrophages and αSMA monoclonal mouse antibody (1:500; DAKO) as a marker for vascular smooth muscle cells and fibroblasts. To detect T lymphocytes CD3 rabbit monoclonal antibody (1:200; DAKO) was used. CD4 and CD8 rat monoclonal antibodies (undiluted, gift from W. Buurman) were used to distinguish between respectively T-helper cells and cytotoxic T-cells. Sirius red staining was used to detect collagen content. Morphometric analyses were performed using a Leica Quantimet with Qwin3.5.1 software (Leica Microsystems). Fluorescent immunohistochemistry was used to determine the presence of CD11c+ cells in the aortic lesions. CD11c, CD11b, CD4 and CD8 antibodies (all BD Biosciences) were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophyll protein (PerCP). Sections were analyzed with a Leica TCS SP5 multi-photon microscope (Leica, Germany).

**Flow Cytometry**

Spleen and lymph nodes were harvested from donor mice and single cell suspensions were prepared and stained with anti-DX5, -TCR, -CD3, -CD4, -CD8, CD25, FoxP3, -CD44, -CD62L, -CD11c (BD Biosciences) or isotype control IgG (PharMingen). Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP). Cells were analyzed using a FACS-Canto II (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).
Chapter 5

**Cytokine production**
Spleen and lymph nodes from ApoE/⁻ and DNR mice treated (n=5) or mDCs (n=6) were isolated and gently mashed through a 70 μm nylon cell strainer. Red blood cells were lysed using 0.83% NH₄Cl in 0.01 M tris/HCL, pH 7.2. Splenocytes and lymph node cells were cultured in triplicate at 3x10⁵ cells/well in the presence of soluble αCD3/CD28 (2 μg/ml) in cRPMI. 48 Hours later, supernatants were collected and TGF-β, IFN-γ, IL-10, IL-12p70, IL-4, IL-17 were measured. Cytokine levels were determined in undiluted supernatant following the manufacturer’s instructions. (All ELISA’s were purchased at Ebioscience, Belgium).

**Statistical analysis**
Values are expressed as mean ± SEM. Data were analyzed by either a two-tailed Student’s T test or a non-parametric Mann-Whitney U-test. Statistical analysis was performed using Prism software. Probability values of P<0.05 were considered significant.
Results

General
At 20 weeks of age, no significant differences were observed between ApoE\textsuperscript{−/−} CD11cDNR mice and ApoE\textsuperscript{−/−} mice with regard to body weight. Decreased levels of total plasma-cholesterol were detected in ApoE\textsuperscript{−/−}CD11cDNR animals compared to ApoE\textsuperscript{−/−} controls (ApoE\textsuperscript{−/−}CD11cDNR 5.97±1.25 mg/dl vs ApoE\textsuperscript{−/−} 10.10±0.78 mg/dl; $P<0.05$) while serum triglycerides (TG) did not differ between both groups (ApoE\textsuperscript{−/−}CD11cDNR 2.33±0.24 mg/dl vs ApoE\textsuperscript{−/−} 2.57±0.29 mg/dl). In addition, autopsy of > 20 organs revealed no abnormalities or pathologies.

Localisation and subtype of CD11c\textsuperscript{+} cells in atherosclerotic plaques
To detect whether CD11c\textsuperscript{+} cells are present in atherosclerotic plaques, where they were localized and which plaque cell types express CD11c, aortic roots of both ApoE\textsuperscript{−/−}CD11cDNR and ApoE\textsuperscript{−/−} mice were double labelled CD11c and cell-type specific markers. Using 2-photon microscopy we were able to clearly distinguish between the different CD11c\textsuperscript{+} expressing cell types (Figure 1).

CD11c positive cells were commonly present in the shoulder region of the plaque and in the adventitia. Interestingly, only a small percentage of CD11c\textsuperscript{+} cells expressed CD11b. This indicates that the majority of CD11c\textsuperscript{+} cells in the plaque were dendritic cells (DC), which was confirmed by double staining with CD11c, CD4 and/or CD8, or macrophage foam cells which are also known to be CD11c\textsuperscript{+}CD11b\textsuperscript{−} but not NK-cells (Figure 1).

Figure 1: Multi-photon immunohistochemistry to determine the presence of CD11c\textsuperscript{+} cells in the aortic lesions.
(a) CD11c-FITC and CD11b-PE. Both cell types are clearly present in both lesion and adventitia. Only scarce double positive cells are present. (b) Detail of adventitia where massive infiltration of CD11c positive cells (FITC) is observed. (c) Detail of the atherosclerotic lesion. CD11c positive cells (FITC) together with CD11b positive cells (PE) are present in the shoulder region of the plaque.
Defective TGF-β signaling in CD11c+ cells accelerates atherosclerosis and enhances the inflammatory state of the lesion

ApoE−/− mice with disrupted TGF-β signaling in CD11c+ cells (ApoE−/−CD11cDNR) exhibited a 2-fold increase in atherosclerotic plaque area in the aortic root (ApoE−/−CD11cDNR 2.37×10^5±5.33×10^4 μm² vs ApoE−/− 1.11×10^5±2.09×10^4 μm², n=8/group, P<0.05) (Figure 2). Moreover, the percentage of CD45+ leukocytes was drastically increased in plaques of ApoE−/−CD11cDNR animals compared to controls (ApoE−/−CD11cDNR 24.04±6.63% vs ApoE−/− 9.74±3.14% CD45+cells, P<0.05) (Figure 3 A-C).

In order to have a more precise view on which leukocyte subsets were increased, we analyzed the lesions for the presence of different T-cell (CD3, CD4, CD8, FoxP3), and macrophage markers (Moma-2). Plaques of ApoE−/−CD11cDNR mice contained significantly more CD3+ T-cells than the controls (ApoE−/−CD11cDNR 21.71±7% vs ApoE−/− 5.16±1% CD3+cells, P<0.05) (Figure 3 D-F). Increased levels of CD4+ T-helper cells were also found in ApoE−/−CD11cDNR 12.14±2.73% vs ApoE−/− 5.73±1.25% CD4+cells, P<0.05) (Figure 4 A-C). In addition, a substantial amount of CD8+ cytotoxic T-cells was present in plaques of ApoE−/−CD11cDNR mice while CD8+ T-cells were almost absent in the control group (ApoE−/−CD11cDNR 9.06±3.21% vs ApoE−/− 2.13±0.34% CD8+cells, P<0.05) (Figure 4 D-F).
Deficiency of TGF-β signaling in CD11c⁺ cells

Figure 3: Disruption of TGF-β signaling in CD11c⁺ cells enhances plaque inflammation.
Number of CD45 positive cells per total cells number in the atherosclerotic plaque (A). Representative CD45 immuno staining (B and C) (magnification 20x). Number of CD3 positive cells per total cells number in the atherosclerotic plaque (D). Representative CD3 immuno staining (E and F) (magnification 20x) (*P<0.05).

Figure 4: Defective TGF-β signaling in CD11c⁺ cells attracts both CD4⁺ and CD8⁺ T-cells into the lesions.
Number of CD4 positive cells per total cells number in the atherosclerotic plaque (A). Representative CD4 immuno staining (B and C) (magnification 20x). Number of CD8 positive cells per total cells number in the atherosclerotic plaque (D). Representative CD8 immuno staining (E and F) (magnification 20x) (*P<0.05).
However, no differences in FoxP3 staining could be observed between both groups (ApoE⁻/⁻CD11cDNR 1.01x10⁻² ±0.53x10⁻² % vs ApoE⁻/⁻ 0.87x10⁻² ±0.37x10⁻² % FoxP3⁺cells). In addition macrophage (Moma-2) content did not differ between both groups (ApoE⁻/⁻CD11cDNR 19.10±4.42% vs ApoE⁻/⁻ 21.08±2.41% Moma-2⁺cells).

**Defective TGF-β signaling in CD11c⁺ cells inhibits plaque fibrosis**

Collagen content on Sirius red-stained sections revealed significantly lower amounts of collagen in plaques of ApoE⁻/⁻CD11cDNR mice than in ApoE⁻/⁻ mice (ApoE⁻/⁻CD11cDNR 30.21±2.15 % vs ApoE⁻/⁻ 37.96±2.66 % Sirius Red⁺ area, \( P < 0.05 \) ) (Figure 5 A-C). The low plaque collagen content was accompanied by a decrease in α-smooth muscle cell⁺ (ASMA) content (ApoE⁻/⁻CD11cDNR 4.02±0.71 % vs ApoE⁻/⁻ 9.46±1.83 % ASMA⁺ area, \( P < 0.05 \) ) (Figure 5 D-F). These results demonstrate that TGF-β receptor deficiency in CD11c⁺ cells reduces plaque fibrosis, indicative of plaque destabilization.

**Figure 5: Defective TGF-β signaling in CD11c⁺ cells leads to an unstable plaque phenotype.**
Percentage of Sirius red positive staining (A). Representative Sirius red staining (B and C) (magnification 20x). Amount of αSMC content in the atherosclerotic plaque (C). Representative ASMA staining (D and E) (magnification 20x) (*\( P < 0.05 \)).
Deficiency of TGF-β signaling in CD11c+ cells

Defective TGF-β signaling in CD11c+ cells alters T-cell homeostasis

To check whether the increased T-cell content in the ApoE-/-CD11cDNR lesions was due to an altered T-cell homeostasis, we used FACS analysis to determine the expression of homing receptors CD44 and CD62L on CD4+ and CD8+ T-cells in the spleen to distinguish from naïve (CD44lowCD62Lhigh) or effector-memory (CD44highCD62Llow) phenotype (Figure 6). The CD4+ T-cell population of the ApoE-/-CD11cDNR group had a significant decrease in the marker for naïve T-cells (CD4+ CD44lowCD62Lhigh: ApoE-/- 52.96±4.46 % vs ApoE-/-CD11cDNR 24.39±2.71 %; \( P < 0.05 \)) and an increase in the marker for effector memory T-cells (CD4+ CD44highCD62Llow population: ApoE-/- 45.85±4.40 % vs ApoE-/-CD11cDNR 73.07±2.22 %; \( P < 0.05 \)). Similar effects were seen in the CD8 population (CD8+ CD44lowCD62Lhigh population: ApoE-/- 81.66±2.50 % vs ApoE-/-CD11cDNR 59.50±5.98 %; \( P < 0.05 \); CD8+ CD44highCD62Llow population: ApoE-/- 17.21±2.43 % vs ApoE-/-CD11cDNR 35.46±4.42 %; \( P < 0.05 \)). CD62L surface expression is lost when cells become activated. This means that both CD4+ T-helper cells and CD8+ cytotoxic T-cells from ApoE-/-CD11cDNR have characteristics of differentiated T-cells. These effector T-cells, in contrast to naïve T-cells, are potent to enter inflamed tissues.

![Figure 6: Loss of TGF-β signaling in CD11c+ cells leads to the generation of effector T-cells in spleen.](image)

Percentage of naïve CD4+ T-cells (A). Percentage of naïve CD8+ T-cells (B). Percentage of effector CD4+ T-cells (C). Percentage of effector CD8+ T-cells (D) (*\( P < 0.05 \)).
Chapter 5

In addition to T-cell differentiation, an enhanced CD4/CD8 ratio was observed in the spleen of CD11cDNR animals (ApoE−/- 1.42±0.09 % vs ApoE−/-CD11cDNR 1.826±0.14 %  P<0.05) (Figure 7 A-C). Finally, a significant increase was observed in the amount of CD25+FoxP3+ double positive cells within the CD4+ population (Tregs) in the spleen of ApoE−/-CD11cDNR animals (ApoE−/- 9.16±0.65 % vs ApoE−/-CD11cDNR 15.33±1.33 %;  P<0.05) (Figure 7 D).

Figure 7: Inhibition of TGF-β signaling in CD11c+ cells disturbs T-cell homeostasis in spleen.
Percentage of CD3+CD4+ T-cells (A). Percentage of CD3+CD8+ T-cells (B). CD4/CD8 ratio T-cells (C). Percentage CD25+FoxP3+ of CD4+ T-cells (D) (*P<0.05).

TGF-β deficiency in CD11c+ cells is associated with increased levels of Th1, Th2 and Th17 cytokines

To determine whether the observed T-cell phenotype correlated with enhanced cytokine levels, T-cell cytokines were determined in the spleen after re-stimulation in vitro with aCD3/CD28. An 8-fold increase of the Th1 cytokine IFN-γ was detected in the ApoE−/-CD11cDNR group (ApoE−/-CD11cDNR 1370.61±534.34 pg/ml vs ApoE−/- 171.17±70.5 pg/ml,  P<0.05) (Figure 8 A). Furthermore, significant increases were found in Th2 cytokines IL-4 and IL-10 (IL-4: ApoE−/-CD11cDNR 161.00±29.99 pg/ml vs ApoE−/- 71.00±10.53 pg/ml,  P<0.05; IL-10: ApoE−/-CD11cDNR 500.00±80.26 pg/ml vs ApoE−/- 346.67±13.14 pg/ml,  P<0.05) (Figure 8 B and C). Finally, a 5-fold increase in IL-17 levels was detected (ApoE−/-CD11cDNR 101.78±22.81 pg/ml vs ApoE−/- 20.93±1.73 pg/ml,  P<0.05) (Figure 8D). In addition to cytokine levels, T-cell proliferation was measured but no significant differences could be observed in T-cell proliferation (data not shown).
Deficiency of TGF-β signaling in CD11c+ cells

Inhibition of TGF-β signaling does not affect DC maturation

Since the net outcome of T-cell immune responses is influenced by the activation stage of antigen-presenting cells, we analyzed the maturation status of the CD11c+ DCs in spleen. TGF-β is known to inhibit critical co-stimulatory molecules on the surface of DCs and therefore regulate DC maturation. However, analysis of cell surface markers of dendritic cells showed that DCs from ApoE−/−CD11cDNR animals had a differentiation process similar to ApoE−/− DCs. No differences could be observed in MHC-II, CD86 or CD40 expression (Figure 9).

Figure 8: Disruption of TGF-β signaling in CD11c+ cells increases cytokine expression.

Concentrations (pg/ml) of IFN-γ (A), IL-10 (B), IL-4 (C), and IL-17 (D) in supernatants of cultured splenocytes, stimulated with aCD3/CD28 (*P<0.05).

Inhibition of TGF-β signaling does not affect DC maturation

Since the net outcome of T-cell immune responses is influenced by the activation stage of antigen-presenting cells, we analyzed the maturation status of the CD11c+ DCs in spleen. TGF-β is known to inhibit critical co-stimulatory molecules on the surface of DCs and therefore regulate DC maturation. However, analysis of cell surface markers of dendritic cells showed that DCs from ApoE−/−CD11cDNR animals had a differentiation process similar to ApoE−/− DCs. No differences could be observed in MHC-II, CD86 or CD40 expression (Figure 9).
In the present study, we highlighted the importance of TGF-β signaling in cells of the innate immune system in the progression of atherosclerosis. When TGF-β signaling in CD11c+ cells (i.e. monocytes, dendritic cells, macrophages and NK-cells)\textsuperscript{28, 32} was abrogated, atherosclerotic plaque size increased significantly. Moreover, plaques contained increased amounts of CD4+ and CD8+ T-cells and a low percentage of collagen, reminiscent of a rupture prone plaque in humans.

Several studies have provided evidence for an important role for TGF-β as an immune modulating cytokine in atherosclerosis. In patients, lowered serum levels of TGF-β are detected but can be increased with tamoxifen treatment with a consequent cardioprotective effect.\textsuperscript{33} In addition, mutations and reduced expression of the type II receptor gene have both been detected in cells of human atherosclerotic lesions.\textsuperscript{34-36} In mice, systemic inhibition of TGF-β signaling in ApoE\textsuperscript{-/-} mice by using a recombinant soluble TGF-β type II receptor\textsuperscript{23} or a blocking TGF-β1 antibody results in an unstable atherosclerotic plaque phenotype, whereas cardiac overexpression of TGF-β1 (resulting in increased plasma levels of TGF-β) limits plaque growth and induces plaque stabilization (Table 1).\textsuperscript{24} However, the immune modulating effects of TGF-β are not restricted to T-cells. Although TGF-β producing regulatory T-cells

\textbf{Discussion}

In the present study, we highlighted the importance of TGF-β signaling in cells of the innate immune system in the progression of atherosclerosis. When TGF-β signaling in CD11c+ cells (i.e. monocytes, dendritic cells, macrophages and NK-cells)\textsuperscript{28, 32} was abrogated, atherosclerotic plaque size increased significantly. Moreover, plaques contained increased amounts of CD4+ and CD8+ T-cells and a low percentage of collagen, reminiscent of a rupture prone plaque in humans.

Several studies have provided evidence for an important role for TGF-β as an immune modulating cytokine in atherosclerosis. In patients, lowered serum levels of TGF-β are detected but can be increased with tamoxifen treatment with a consequent cardioprotective effect.\textsuperscript{33} In addition, mutations and reduced expression of the type II receptor gene have both been detected in cells of human atherosclerotic lesions.\textsuperscript{34-36} In mice, systemic inhibition of TGF-β signaling in ApoE\textsuperscript{-/-} mice by using a recombinant soluble TGF-β type II receptor\textsuperscript{23} or a blocking TGF-β1 antibody results in an unstable atherosclerotic plaque phenotype, whereas cardiac overexpression of TGF-β1 (resulting in increased plasma levels of TGF-β) limits plaque growth and induces plaque stabilization (Table 1).\textsuperscript{24} However, the immune modulating effects of TGF-β are not restricted to T-cells. Although TGF-β producing regulatory T-cells
Deficiency of TGF-β signaling in CD11c+ cells

are well known to control T-cell activation and differentiation, and to play a crucial role in atherosclerosis, the immune-regulatory capacity of other cell-types is also controlled via TGF-β. Interestingly, it has been reported that phagocytosis of apoptotic cells leads to TGF-β secretion, which inhibits the production of inflammatory cytokines and chemokines in macrophages including IL-1β and TNF-α. In addition, several studies have shown that TGF-β inhibits in vitro activation and maturation of DCs. TGF-β inhibits upregulation of critical co-stimulatory molecules on the DC surface, thereby reducing its antigen-presenting capacity and DC mediated T-cell responses. However these studies focused on Langerhans cell development, and might not be applicable in the present study, where we did not observe an effect on DC maturation in vivo. In this study we proved the importance of TGF-β signaling in CD11c positive cells in atherosclerosis using the CD11c-DNR mouse. In this mouse model, specific expression of dnTGFβRII was found in CD11c expressing cells, including DC subsets and NK cells, whereas no expression was found in NKT cells, T-cells or B-cells. In atherosclerotic plaques, an important local and systemic role for CD11c expressing cells has been reported recently. In conditions of hypercholesterolemia, monocytes expressed high levels of CD11c, and show increased adherence to the arterial wall. In CD11c-/-/ApoE-/- mice, monocyte recruitment was impaired and only small atherosclerotic plaques developed. This was also observed in the atherosclerotic plaque, where CD11c+ cells with foam cell morphology also were positive for Moma-2, a macrophage marker. Cho et al. studied gene expression profiles during foam cell formation and confirmed that foam cells develop a dendritic cell-like phenotype accompanied by CD11c expression. Moreover, CD11c plays a crucial, but yet unidentified role in cholesterol homeostasis. When there is an expansion of CD11c+ cells, cholesterol levels and the amount of atherosclerosis decreases, while a depletion of CD11c+ cells results in hypercholesterolemia and an increase in atherosclerosis. In the present study we observed decreased levels of cholesterol in the plasma of ApoE-/-CD11cDNR mice. Recently it has been shown that depletion of DCs resulted in enhanced cholesteremia suggesting a close relationship between the DC population and plasma cholesterol levels. Moreover, Frutkin et al. described that hypercholesterolemia induces TGF-β expression. Since in our study we blocked TGF-β signalling in DCs, our data may suggest an interaction between TGF-β, DCs and the cholesterol metabolism. However, the reason why the reduced cholesterol levels did not result in reduced lesion size remains unclear.

In the present study, we found that TGF-β signaling in CD11c+ cells is involved in the recruitment of inflammatory cells into the plaque, as well as in maintaining T-cell homeostasis. Lack of TGF-β signaling in CD11c+ cells results in massive infiltration of CD4+ and CD8+ cells in atherosclerotic plaques and in a systemic expansion of CD44hiCD62Llo T-cells. These data demonstrated that blockage of TGF-β signaling in CD11c cells lead to a shift from naïve T-cells towards effector (CD44hiCD62Llo) T-cells. This effector-T-cell phenotype explained the influx of large amounts of CD4+ and CD8+ T-cells into the lesions, since naïve T-cells are not capable of infiltrating inflamed tissues. In addition, elevated levels of IFN-γ, TNF-α, IL-4, IL-10, IL-17 were detected. This broad array of expressed cytokines refers to both Th1 and Th2 subsets. The effect on atherosclerosis can therefore
not be explained by a shift in the Th1/Th2 balance. In addition, high levels of IL-17 were detected in the CD11cDNR animals, referring to an enhanced Th17 phenotype as well. These results are in concordance with Laouar et al. who studied the CD11cDNR mouse in experimental autoimmune encephalomyelitis (EAE) were an increase in inflammation in the CNS is accompanied with increased levels of Th1 and Th17 cytokines.\(^42\) In addition abrogation of IL-17 receptor signaling in LDLr\(^{-/-}\) mice attenuated atherosclerosis.\(^43\) Moreover, Th17 cells have been identified in patients with acute coronary syndrome.\(^44\) Taken together these data suggest a potential proatherogenic role. We therefore hypothesize that interruption of TGF-\(\beta\) signaling in CD11c\(^+\) cells results in altered T-cell priming by DCs. In conclusion, we state that TGF-\(\beta\) signaling in CD11c expressing cells is fundamental in the immunomodulation during atherogenesis. We showed that impaired TGF-\(\beta\) signalling in CD11c\(^+\) cells resulted in an increase of activated effector T-cell which can more easily migrate into the atherosclerotic lesions with a consequential development of plaques, which are high in inflammation and low in fibrosis. We therefore provided a first inhibitory link between the innate and adaptive immune response with an important anti-inflammatory function for TGF-\(\beta\) signaling in CD11c\(^+\) cells.
Deficiency of TGF-β signaling in CD11c+ cells

Table 1: Overview of studies on the effects of TGF-β signaling in atherosclerosis, with focus on plaque area, plaque phenotype and the induced cytokines.

<table>
<thead>
<tr>
<th>Group (Year)</th>
<th>Reference</th>
<th>Experiment</th>
<th>Plaque area</th>
<th>Plaque inflammation</th>
<th>Plaque fibrosis</th>
<th>Induced cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mallat (2001)</td>
<td>24</td>
<td>Abs against TGF-β1, TGF-β2 and TGF-β3 ApoE/− acceptor mice</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lutgens (2002)</td>
<td>23</td>
<td>rs TGF-βRII ApoE/− acceptor mice</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Robertson (2003)</td>
<td>26</td>
<td>CD4-dnTGF-βRII tg+ ApoE/− background</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>increased IFN-γ, TNF-α IL-4,5, 10</td>
</tr>
<tr>
<td>Frutkin (2009)</td>
<td>25</td>
<td>Cardiac specific TGF-β overexpression ApoE/− background</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>decreased IFN-γ TNF-α, MIP-1α</td>
</tr>
</tbody>
</table>

(Ref: Reference; BMT: Bone Marrow Transplantation; +: increase; - : decrease)


1 Division of Biopharmaceutics, LACDR, Leiden, The Netherlands
2 Dept. of Rheumatology, LUMC, Leiden, The Netherlands