Abrogated transforming growth factor beta receptor II (TGFβRII) signalling in dendritic cells promotes immune reactivity of T cells resulting in enhanced atherosclerosis

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Aims

The importance of transforming growth factor beta (TGFβ) as an immune regulatory cytokine in atherosclerosis has been established. However, the role of TGFβ signalling in dendritic cells (DCs) and in DC-mediated T cell proliferation and differentiation in atherosclerosis is unknown.

Methods and results

Here, we investigated the effect of disrupted TGFβ signalling in DCs on atherosclerosis by using mice carrying a transgene resulting in functional inactivation of TGFβ receptor II (TGFβRII) signalling in CD11c+ cells (Apoe−/−CD11cDNR). Apoe−/−CD11cDNR mice exhibited an over two-fold increase in the plaque area compared with Apoe−/− mice. Plaques of Apoe−/−CD11cDNR mice showed an increase in CD45+ leucocyte content, and specifically in CD3+ , CD4+ and CD8+ cells, whereas macrophage content was not affected. In lymphoid organs, Apoe−/−CD11cDNR mice had equal amounts of CD11c+ cells, and CD11c+CD8− and CD11c+CD82 subsets, but showed a subtle shift in the CD11c+CD82 population towards the more inflammatory CD11c+CD8−CD4+ DC subset. In addition, the number of plasmacytoid-DCs decreased. Maturation markers such as MHCII, CD86 and CD40 on CD11chi cells did not change, but the CD11cDNR DCs produced more TNFa and IL-12. CD11c+ cells from CD11cDNR mice strongly induced T-cell proliferation and activation, resulting in increased amounts of effector T cells producing high amounts of Th1 (IFN-γ), Th2 (IL-4, IL-10), Th17 (IL-17), and Treg (IL-10) cytokines.

Conclusion

Here, we show that loss of TGFβRII signalling in CD11c+ cells induces subtle changes in DC subsets, which provoke uncontrolled T cell activation and maturation. This results in increased atherosclerosis and an inflammatory plaque phenotype during hypercholesterolaemia.

Keywords

Atherosclerosis • Inflammation • TGFβ • Dendritic cell

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Introduction

The adaptive immune system plays a crucial role in atherosclerosis. Different T cell subsets, B cells, and natural killer (NK) T cells modulate atherosclerotic plaque progression, plaque inflammation, and athero-thrombosis. 1 The polarization of the immune response determines whether a response is pro-atherogenic or anti-atherogenic: differentiation of T cells along the Th1-pathway are pro-atherogenic, along the Treg pathway are anti-atherogenic, and along the Th2 and Th17 pathway either pro- or anti-atherogenic. 1

Dendritic cells (DCs) have a central role in initiating and regulating immune responses and especially T cell responses, 2 both in lymphoid and peripheral tissue.

It has become evident that many distinctive DC subtypes exist, each with a particular location, a specialized function in the immune system and expressing a unique set of cell surface markers. 3,4 Commonly, DCs are classified in the CD11c+ MHCII+ conventional DCs (cDCs) and the non-conventional DCs. cDCs are subdivided into migratory and lymphoid-tissue-resident DCs (which can be further divided into two subsets; the CD8+ cDC and the CD8− cDC). 5 The non-conventional DCs consist of the plasmacytoid (p)DCs and monocyte-derived DCs. Plasmacytoid DCs are found in many tissues including blood, thymus, bone marrow, liver, and lymphoid organs. Plasmacytoid DCs are known to secrete large amounts of type I interferons (IFNs) and have been associated with the preservation of peripheral tolerance and with the initiation of autoimmune responses. 3 However, the specific role of these distinct DC subsets in atherosclerosis remains to be elucidated. 6

In the vasculature, DCs are present in the arterial intima 7 and were recently ascribed an immune-modulatory role in atherosclerosis. 8 Intimal DCs are able to accumulate lipid. 9 In more advanced stages of atherosclerosis, DCs were found to affect plaque growth as well as cholesterol homeostasis. Hypercholesterolaemic mice containing DCs with an enhanced lifespan and immunogenicity as well as cholesterol homeostasis. Hypercholesterolaemic mice containing DCs with an enhanced lifespan and immunogenicity (DC-8Cl2ApoE−/− DC-8Cl2LDLr−/− mice) had increased Th1 and Th17 cytokine expression levels, but decreased plasma cholesterol levels, and no change in the atherosclerotic plaque area. 8 In contrast, ApoE−/− mice in which DCs were depleted (CD11c-DTRApoE−/−) mice had increased cholesterol levels. 10 Vaccination using mature DCs pulsed with oxidized low-density lipoprotein (oxLDL) reduced lesion size, 11 whereas vaccination with malondialdehyde (MDA)-LDL pulsed DCs aggravated atherosclerosis, 12 illustrating the tight and complex modulation of atherosclerosis by DCs in different conditions.

Other studies stressing the importance of DC-T cell interactions in atherosclerosis are interventions in co-stimulatory molecules such as CD80/86, CD40/CD40L, OX40L/OX40, CD137 and ICOS, which are all pivotal for DC-mediated T cell proliferation and polarization. 13–18 For example, we have shown that inhibition of CD40L, CD40, or CD40-TNF-Receptor Associated Factor (TRAF) 6 interactions reduces atherosclerosis by affecting T cell activation, polarization, and monocyte activation, and by upregulating the anti-inflammatory cytokine transforming growth factor beta (TGFβ). 19–21

Transforming growth factor beta is a powerful modulator of immune responses. It not only regulates immune responses by inhibiting the proliferation of naïve and activated T cells, and by mediating T cell polarization, but also has a crucial role in mediating DC functions. 22,23 Transforming growth factor beta can immobilize DCs, induce DC apoptosis, and can decrease CD40, CD80, and CD86 expression on the cell surface. Moreover, TGFβ can dampen tumour necrosis factor (TNF)α, IL12, and CCL5 production in DCs, thereby rendering a more tolerogenic DC that promotes Treg development. 24

In the present study, we investigated the effects of TGFβRII signalling in DCs on atherosclerosis in ApoE−/− mice that contained a dysfunctional TGFβ Receptor II in CD11c+ cells (ApoE−/− CD11cDNR). We found that impairment of TGFβRII signalling in CD11c+ cells accelerated atherosclerosis, enhanced the influx of both CD4+ and CD8+ T cells into the plaque, and decreased plaque fibrosis, while lowering cholesterol levels. This aggravated atherosclerosis was accompanied by augmented pro-inflammatory T cell and DC responses.

Methods

ApoE−/− CD11c-dnTGFβRII transgenic mice

CD11c-dnTGFβRII (CD11cDNR) mice were generated as described previously and were backcrossed to ApoE−/− mice. 25 The transgene was composed of the human TGFβ type II receptor sequence (−7 and +573) that encodes for the extracellular and transmembrane regions, but not the intracellular region of the TGFβ type II receptor, thereby preventing TGFβ signalling. 26 Genotypes were verified by polymerase chain reaction (PCR) as described before and hemizygous transgenes and their littermate wild types (both ApoE−/− mice) had increased Th1 and Th17 CD4+ T cells accelerated atherosclerosis, enhanced the influx of both CD4+ and CD8+ T cells into the plaque, and decreased plaque fibrosis, while lowering cholesterol levels. This aggravated atherosclerosis was accompanied by augmented pro-inflammatory T cell and DC responses.

Histology and morphometry

The plaque area was analysed in the aortic root using serial 6 μm sections 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 haematoxylin and eosin (HE). The plaque area was measured on a Leica DM3000 Light microscope (Leica Microsystems) coupled to a computerized morphometry system (Leica Qwin 3.5.1).

Immunohistochemistry

Consecutive sections were immunolabelled with anti-CD45 rat monoclonal antibody (1:5000; BD Biosciences) to detect all inflammatory cells, anti-Moma-2 rat monoclonal antibody (1:50; Serotec) to detect macrophages, anti-αSMA monoclonal mouse antibody (1500; DAKO) as a marker of vascular smooth muscle cells and myofibroblasts and anti-MMP9 goat polyclonal antibody (1:200; Santa Cruz) to detect matrix metalloproteinase 9. Anti-CD3 rabbit monoclonal antibody (1:200; DAKO) was used to detect T lymphocytes, and anti-CD4 and anti-CD8 rat monoclonal antibodies (undiluted, gift from W. Buurman, Department of General Surgery, Academic
Hospital Maastricht) to distinguish between T-helper cells and cytotoxic T-cells, respectively. Sirius red staining was used to detect collagen content, both by brightfield- and polarization light microscopy. 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, DX5, CD4, and CD8 antibodies (all BD Biosciences) were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP). Sections were analysed with a Leica TCS SP5 multi-photon microscope (Leica).

**Lipids and lipoproteins**

Plasma cholesterol and triglyceride levels were measured using colorimetric assays (Roche). Size fractionation of lipoproteins was performed by fast-performance liquid chromatography (FPLC) using a 30 × 0.32 cm Superose 6b micro-FPLC column (GE Healthcare) followed by in-line cholesterol detection, as described previously.26

**Antibody measurements**

Antibody (Ab) titres to Cu2+-LDL and MDA-LDL were measured in the plasma as previously described.27 In brief, copper-oxidized LDL (CuOx-LDL) and malondialdehyde-modified LDL (MDA-LDL) were generated from freshly isolated human LDL. Binding of specific IgM, IgG1, and IgG2c antibodies in individual plasma samples to coated antigens were measured by chemiluminescent enzyme-linked immunosorbent assay (ELISA) at indicated dilutions. Bound antibodies were detected using alkaline phosphatase (AP)-conjugated goat-anti-mouse IgM or biotinylated goat-anti-mouse IgG1 and goat-anti-mouse IgG2c (Jackson Immuno Research) followed by AP-conjugated Neutravidin (Thermo Scientific).

**Real-time polymerase chain reaction**

RNA was isolated from cultured bone marrow-derived macrophages using the RNeasy Mini kit II (Qiagen). One microgram of total RNA was reverse transcribed into cDNA using the SuperScript™ VIREMT cDNA Synthesis Kit (Invitrogen). Real-time PCR reactions (7900HT Fast Real Time PCR system, Applied Biosystems) were carried out with cDNA (equivalent to 10 ng total RNA), TaqMan™ Fast Advanced Master Mix, and TaqMan™ Gene Expression assays (all Applied Biosystems) for CD40, CD86, TNFα, MHCII, Mannose receptor, Arginase 1, RELMα, and, Ym-1 according to the instructions of the manufacturer. Samples were assayed in quadruplicates. The mRNA expression was normalized to that of the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression.

**Flow cytometry**

Spleen and lymph nodes were harvested from old and single cell suspensions were prepared and stained with anti-TCR, -CD3, -CD4, -CD8, -CD25, -Foxp3, -CD44, -CD62L, -CD11c, -CD40, -MHCII, -CD86, -Ly6C, -CD19 (BD Biosciences), or isotype control IgG (all BD Biosciences). Antibodies conjugated to FITC, PE, allophycocyanin (APC), or PerCP were used and cells were analysed using a FACS-Canto II (BD Biosciences).

**Cell culture**

Spleen and lymph nodes from Apcε–/– and Apcε+/+ CD11c-DNR mice (n = 5) were isolated and gently mashed through a 70 μm nylon cell strainer. Splenocytes and lymph node cells were cultured in triplicate at 3 × 105 cells/well in the presence of soluble anti-CD3/CD28 (2 μg/mL) in complete RPMI (RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FCS, and 100 U/mL streptomycin/penicillin (PAA)). After 48 h, supernatants were collected and TGFβ, IFNγ, IL-10, IL-12p, IL-4, and IL-17 production were measured by ELISA (Bioscience).

**Isolation and purification of CD4+ and CD11c+ cells**

CD4+ cells were negatively selected using of a cocktail of antibody-coated magnetic beads (Miltenyi Biotech), resulting in 92% pure CD4+ T cells. CD11c+ cells were positively selected with vitamin-conjugated anti-CD11c mAb (N418, Pharmingen), streptavidin microbeads (Miltenyi Biotec), followed by two consecutive magnetic cell separations using LS columns (Miltenyi Biotec), resulting in 95% CD11c+ cells.

**Bone marrow-derived dendritic cells/macrophages**

Femurs and tibiae were removed and the ends of the bones were carefully cut off. The bone marrow was flushed out and filtered. Red blood cells were lysed and the cells were counted and adjusted to 0.5 × 106 cells/mL by adding RPMI 1640 containing 10% heat-inactivated FCS, glutamine, 100 U/mL penicillin/streptomycin, and 50 μM β-Mercaptoethanol. Granulocyte-macrophage colony-stimulating factor (20 ng/mL) was added to generate DCs. At day 10, 99% of the cells were CD11c+. A similar protocol was used to obtain macrophages where L929-conditioned medium (M-CSF) was added.

**Cell culture, proliferation, and cytokine assays**

Cells were cultured in RPMI 1640 supplemented with Glutamax, 10% FCS, 0.02 mM β-mercaptoethanol, and antibiotics. For cytokine measurements, bone marrow-derived CD11c+ cells (2 × 106 cells/well) were stimulated with LPS (10 μg/mL) and IFNγ (100 U/mL) for 48 h. TNFα and IL-12 production in the supernatants were measured using specific ELISAs (R&D Systems). CD4+ T cells were cultured at 1 × 105 cells/well for 48 h in anti-CD3-coated microplates (10 μg/mL) either alone or with CD11c+ cells (2 × 105 cells/well). Cytokine production in the supernatants was measured using specific ELISAs (R&D Systems). For proliferation, cells were cultured at 37°C for 72 h and pulsed with 1 μCi of [3H] thymidine (Amersham) for the last 18 h of culture. Thymidine incorporation was assessed using a TopCount NXT scintillation counter (Perkin Elmer).

**Statistical analysis**

Values are expressed as mean ± SEM. A non-parametric Mann-Whitney U test (two-tailed), or, when appropriate, a student’s t-test was performed (two-tailed). Statistical analysis was performed using Prism (GraphPad). Probability values of P < 0.05 were considered significant.

**Results**

**Localization and subtype of CD11c+ cells in atherosclerotic plaques**

Previously, Laouar et al.28 found specific expression of the DNR transgene in CD11c expressing DCs and NK cells but not in T cells, NKT cells, or B cells. To assess which cells express CD11c (and thus the construct) in atherosclerotic lesions, plaques of 

3719

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**TGFβRII signalling in dendritic cells**
both Apoe−/− CD11cDNR and Apoe−/− mice were double stained to determine the presence, localization, and identity of CD11c+ cells (Figure 1). CD11c+ cells were found in the shoulder region of the plaque and in the adventitia (Figure 1A–C). Interestingly, only a small percentage of CD11c+ cells in the plaque expressed CD11b (monocytes/macrophages), indicating that the majority of CD11c+ cells in the plaque were either DCs, or macrophage-derived foam cells (CD11c+ CD11b−). Interestingly, CD11c+CD8− DCs, which are well known for the production of IL-12 and their capacity for cross presentation, were only occasionally observed in the atherosclerotic lesions (see Supplementary material online, Figure S1A–C). CD11c+ DX5+ NK cells were not identified (data not shown).

**Disruption of TGFβRII signalling in CD11c+ cells affects DC subset distribution and DC function in atherosclerosis**

Apoe−/− CD11cDNR mice and their Apoe−/− littermates had equal amounts of CD11c+ cells in spleen and lymph nodes (Figure 2A), with similar CD11c+CD8+ (Figure 2B) and CD11c+CD8− (Figure 2C) DC fractions (for gating strategies see Supplementary material online, Figure S2). Interestingly, within the CD8− DC subset, the CD8−CD4+ subset, generally thought to have a more pro-inflammatory capacity, prevailed in the Apoe−/− CD11cDNR mice (Figure 2D). Indeed, TNFα and IL12 production were increased in in vitro matured and stimulated CD11cDNR DCs (Figure 2E and F). Plasmacytoid DC (CD3−CD19+CD11c+Ly6c+Ly6chigh) levels were significantly decreased in the Apoe−/−CD11cDNR animals (Figure 2G). Remarkably, maturation markers such as MHCII, CD86, and CD40 were similar (see Supplementary material online, Figure S3A–C).

Bone marrow-derived CD11c+ cells from CD11cDNR mice strongly induced T cell proliferation when co-cultured with T cells from wild-type mice upon stimulation with CD3/CD28 antibodies (Figure 3A). In addition, CD11cDNR splenocytes (Figure 3B–E) or splenic DCs co-cultured with T cells (Figure 3F and G) stimulated with CD3/CD28 resulted in strong activation of T cells that produced high levels of Th1 (increased IFN-γ), Th2 (increased IL-4), Th17 (increased IL-17), and Treg (increased IL-10) associated cytokines, compared with splenocytes and DCs from Apoe−/− littermates.

In Apoe−/− CD11cDNR mice, lymphoid organs showed a slightly increased CD4+CD8+ ratio (Figure 4A) and an increased percentage of CD25+Foxp3+ Tregs within the CD4+ population (Figure 4B). Remarkably, the Apoe−/− CD11cDNR had less naive T cells (CD45R0+CD62L+), and more effector memory T cells (CD45R0CD62Llow), within both the CD4+ and CD8+ subset, suggesting increased mobilization of mature, activated T cells in the absence of TGFβRII-signalling (Figure 4C–F).

**Anti-malondialdehyde-LDL or oxLDL antibodies**

We next tested whether deficiency of TGFβRII signalling in DCs would change antibody titres or subtypes of IgG antibodies directed against modified LDL in the plasma of Apoe−/− Cd11cDNR mice. However, no significant differences in plasma titres of IgG1, IgG2b, IgG2c, or IgM antibodies against Cu²⁺-oxLDL and MDA-LDL could be detected between the two groups of mice (Figure 4G).

**Deficient transforming growth factor beta receptor II signalling in dendritic cells accelerates atherosclerosis and promotes T cell influx in atherosclerotic plaques**

At sacrifice, body weight of Apoe−/− and Apoe−/− CD11cDNR mice was similar. Surprisingly, Apoe−/− CD11cDNR mice had decreased total plasma cholesterol levels, particularly in the...
chylicorn remnant/VLDL fraction, while plasma triglycerides did not differ (see Supplementary material online, Figure S4).

Despite the decrease in total cholesterol levels, Apoe<sup>−/−</sup> CD11cDNR mice exhibited a two-fold increase in atherosclerotic plaque area in the aortic root (Figure 5A, C, D) and a two-fold increase in the percentage of stenosis (Figure 5B) compared with the Apoe<sup>−/−</sup> littermates. Plaque CD45<sup>+</sup> leucocyte content was strongly increased in Apoe<sup>−/−</sup> CD11cDNR animals compared with controls (Figure 6A–D), consistent with the increase in effect-or memory T cells (Figure 4). To more precisely define the actual leucocyte subsets that were increased in Apoe<sup>−/−</sup> CD11cDNR mice, we analysed the lesions for the presence of different T cell (CD3, CD4, CD8, Foxp3), and macrophage markers (Moma-2). Plaques of Apoe<sup>−/−</sup> CD11cDNR mice contained significantly more CD3<sup>+</sup> T cells than the controls (Figure 6E–H). Interestingly, increased levels of both CD4<sup>+</sup> T-helper cells and CD8<sup>+</sup> cytotoxic T cells were found in plaques of Apoe<sup>−/−</sup> CD11cDNR mice (Figure 6I–P). No differences in the number of Foxp3<sup>+</sup> cells could be observed, which was not surprising considering the low numbers of Foxp3<sup>+</sup> cells present in plaques (Apoe<sup>−/−</sup> 1.37 × 10<sup>−5</sup> ± 5.51 × 10<sup>−6</sup> vs. Apoe<sup>−/−</sup> CD11cDNR 5.04 × 10<sup>−6</sup> ± 2.30 × 10<sup>−6</sup> FoxP3<sup>+</sup>cells/μm<sup>2</sup>).

**Absence of transforming growth factor beta receptor II signalling in CD11c<sup>+</sup> cells does not significantly alter macrophage phenotype**

Plaque macrophage (Moma-2) content did not differ between both groups (Apoe<sup>−/−</sup> 21.08 ± 2.41 vs. Apoe<sup>−/−</sup> CD11cDNR

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**Figure 2** Inhibition of transforming growth factor beta signalling in CD11c<sup>+</sup> cells leads to a shift in dendritic cell subsets. Flow cytometric analysis of splenic cells from Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> CD11cDNR mice (n = 5, *p < 0.05). (A) Percentage of CD11c<sup>high</sup> cells (% of non-CD3<sup>+</sup> CD19<sup>+</sup> cells). (B) Percentage of CD8<sup>+</sup> dendritic cells (% of CD11c<sup>high</sup> cells). (C) Percentage of CD8<sup>+</sup> dendritic cells (% of CD11c<sup>high</sup> cells, n = 5, *p < 0.05). (D) TNFα and IL-12 cytokine production by CD11c<sup>+</sup> cells purified from spleen, stimulated by LPS, measured by ELISA (pg/mL). Disruption of transforming growth factor beta signalling in CD11c<sup>+</sup> cells does not alter dendritic cell maturation in vivo. (G) MHCII, (H) CD86, (I) CD40 of Apoe<sup>−/−</sup> and Apoe<sup>−/−</sup> CD11cDNR splenocytes.
19.10 ± 4.42; % Moma-2⁺ positive area in the lesion), and, only a slight alteration in macrophage phenotype was detected. Bone marrow-derived macrophages from Apoe⁻/⁻ CD11cDNR mice stimulated with oxLDL showed increased expression of CD40 and MHCII, but not CD80 or CD86 by flow cytometry (see Supplementary material online, Figure S3D–G). Using real-time PCR, we could not detect any significant changes in expression of TNFα, CD206, Arginase-1, or YM1 (see Supplementary material online, Figure S3H). These data suggest that the absence of TGFβ signalling in CD11c⁺ cells does not affect macrophage phenotype or alters the M1/M2 balance, but rather affects DC and T cell populations.

**Atherosclerotic lesions of Apoe⁻/⁻ CD11cDNR mice exhibit increased matrix turnover**

In atherosclerotic lesions of Apoe⁻/⁻ CD11cDNR mice, the sirius red positive area had significantly decreased compared with controls (Figure 7A–G). Polarization microscopy confirmed this decrease in collagen content, and revealed a shift towards the red spectrum, specifying thicker and more mature collagen fibres (Figure 7A, D, E), probably reflecting the more advanced stage of the plaques in Apoe⁻/⁻ CD11cDNR mice. This was accompanied by a decrease in α-smooth muscle cell actin⁺ (ASMA) cell...
content in the lesion (Figure 7F–H). MMP9 expression significantly increased in lesions of Apoe<sup>−/−</sup>CD11cDNR mice (Figure 7I–K).

**Discussion**

The present study highlights the importance of TGFβRII signalling in DCs for the progression of atherosclerosis. The current experiments clearly show that the absence of TGFβRII signalling in DCs caused an increase in atherosclerotic plaque size, despite a 50% reduction in plasma cholesterol levels. Moreover, plaques contained more CD4<sup>+</sup> and CD8<sup>+</sup> T cells and less collagen and an increased matrix turnover, and generally were reminiscent of a vulnerable, rupture-prone plaque phenotype in humans.

Several studies have provided evidence for an important role of TGFβ as an immune modulating cytokine in atherosclerosis. Systemic inhibition of TGFβ signalling in Apoe<sup>−/−</sup> mice by using a recombinant soluble TGFβ type II receptor<sup>31</sup> or a blocking TGFβ1 antibody<sup>32</sup> resulted in accelerated atherosclerosis. Lesions exhibited an unstable phenotype that contained low amounts of fibrosis, an increased amount of inflammatory cells, and even intraplaque
hemorrhages. Cardiac overexpression of TGFβ1, resulting in increased plasma levels of TGFβ, limited plaque growth and induced plaque stabilization. These effects have predominantly been attributed to TGFβ signalling in T cells. Indeed, mice with abrogated TGFβ signalling in T cells (Apoe<sup>−/−</sup> CD4-dnTGFβRII) also showed accelerated lesion progression, with plaques containing abundant inflammatory cells paralleled by a decrease in plaque fibrosis. The defect in TGFβRII signalling in T cells increased systemically the amount of Th1 and Th2 cytokines, with profound increases in plasma levels of IFNγ, but also of IL-10 and IL-4. Gojova et al. who transplanted bone marrow from CD2-dnTGFβRII mice into Ldlr<sup>−/−</sup> recipients obtained similar results in plaque phenotype, although plaque area decreased.

The immune modulatory effects of TGFβ are not restricted to T cells. Although TGFβ producing Tregs are well known to control T cell activation and differentiation and play a crucial role in atherosclerosis, the immune-regulatory capacity of other cell types is also controlled via TGFβ. Phagocytosis of apoptotic cells leads to TGFβ secretion, which inhibits the production of inflammatory cytokines and chemokines (including IL-1β and TNFα) in macrophages. Moreover, TGFβ also inhibits macrophage activation and may be involved in polarization towards the M2 phenotype.

However, in our hands, disruption of the TGFβ signalling in CD11c<sup>+</sup> cell did not affect macrophage polarization significantly, although M1 markers like CD40 showed a slight decrease, and expression of M2 markers such as Arginase-1 and the mannose receptor a slight increase.

The exact role of TGFβ in DC biology remains to be elucidated. What is known is that TGFβ can immobilize tumour Langerhans cells and reduces their expression of MHCII, CD86, and CD40 as well as the production of IL-12, TNFα, and CCL5, thereby reducing its antigen-presenting capacity and DC-mediated T cell responses. In DC development, TGFβ accelerates the differentiation of common DC progenitors (CDP) towards conventional CD8<sup>+</sup> and CD8<sup>−</sup> DCs (cDC) and blocks the differentiation into pDCs. Although the majority of these studies focused on Langerhans cells and tumour-associated DCs, these data corroborate our findings. We also observed increased DC cytokine production, which had massive effects on T cell activation and augmented T cell proliferation when TGFβ signalling was abrogated in DCs in an atherosclerosis setting. However, in contrast to earlier findings, inhibition of TGFβ-signalling decreased the number of pDCs significantly. A recent report by Daisormont et al. showed that inhibition of pDCs is associated with plaque
progression and inflammation. Therefore, the observed decrease in pDCs in our mouse model is likely secondary to the increase in plaque area and plaque inflammation.

TGFβ signalling in CD11c+ cells seems also crucial for the recruitment of inflammatory cells into the lesion. A lack of TGFβ signalling in CD11c+ cells results in substantial infiltration of CD4+ and CD8+ cells in atherosclerotic plaques and systemic activation of the T cell compartment as evidenced by an expansion of CD44highCD62Llow effector memory T cells. These activated T cells expressed elevated levels of IFNγ, TNFα, IL-4, IL-17, IL-10, and TGFβ. This broad array of expressed cytokines refers to strong activation of the Th1, Th2, and Th17 T cell subsets. Especially, the Th1 subset is crucial for atherosclerosis, while the Th2 subset can be either pro- or anti-atherogenic. The increase in Th17 cells is rather surprising, since TGFβ was considered to be crucial for a Th17 response. However, Th17 cells can develop independently of TGFβ and the role of Th17 cells in atherosclerosis is still controversially discussed with some groups reporting a pro-atherogenic, and others an anti-atherogenic role for Th17 cytokines. Interestingly, ApoE−/− CD11cDNR mice develop a compensatory Treg response, which was insufficient to prevent the increase in atherosclerosis.

**Figure 6** Disruption of transforming growth factor beta signalling in CD11c+ cells enhances plaque inflammation. (A) CD45+ cells per total cell number in the plaque and (B) CD45+ cells per µm2 plaque area (n = 8, *P < 0.05). (C and D) Representative CD45 immunostaining of ApoE−/− and ApoE−/− CD11cDNR aortic root lesions. (E) CD3+ cells per total cell number in the plaque and (F) number of CD3+ cells per µm2 plaque area (n = 8, *P < 0.05). (G and H) Representative CD3 immunostaining of ApoE−/− and ApoE−/− CD11cDNR aortic root lesions. (I) CD4+ cells per total cell number in the atherosclerotic plaque and (J) CD4+ cells per µm2 plaque area (n = 8, *P < 0.05). (K and L) Representative CD4 immunostaining of ApoE−/− and ApoE−/− CD11cDNR aortic root lesions. (M) CD8+ cells per total cell number in the lesions and (N) CD8+ cells per µm2 plaque area (n = 8, *P < 0.05). (O and P) Representative CD8 immunostaining of ApoE−/− and ApoE−/− CD11cDNR aortic root lesions.
Both CD11c+ cells, as well as TGFβ, play a crucial, but yet unidentified role in cholesterol homeostasis. During expansion of CD11c+ cells, cholesterol levels and the amount of atherosclerosis decreases, while a depletion of CD11c+ cells results in hypercholesterolaemia, suggesting a tight regulation between DC homeostasis and lipid metabolism. Frutkin et al. described that hypercholesterolaemia induces TGFβ expression, and Robertson et al. observed decreased cholesterol values in the plasma of Apoe2/2CD11cDNR mice. Surprisingly, decreased levels of cholesterol, especially in the chylomicron remnant/VLDL fraction, were observed in the plasma of Apoe-/-CD11cDNR mice, suggesting an interaction between TGFβ, DCs, and cholesterol metabolism, which requires further investigation.

In conclusion, we observed that TGFβRII signalling in CD11c expressing cells plays a key role in the regulation of the activation of T cells during atherogenesis. More specifically, our data show...
that TGFβ signalling in DCs modulates the interaction between the innate and adaptive immune response by affecting DC and T cell activation, thus dampening inflammation in atherosclerosis.

Our data substantially contribute to the understanding of TGFβ signalling in atherosclerosis and underline the important role of TGF-β in regulating immune responses in atherogenesis. Many TGFβ agonists have been developed and their efficacy was tested in numerous animal models. However, to apply activation of TGFβ as a therapy for atherosclerosis, a number of obstacles need to be overcome. TGFβ signalling is highly dependent on the environmental milieu and can be either beneficial or detrimental. In neoplastic disease for example, TGFβ suppresses the progression of early tumours, but at more advanced stages of the disease cancer cells start producing TGFβ, which then promotes metastasis. In addition, chronic administration of TGFβ has been reported to induce interstitial fibrosis and hepatic fibrosis. Targeted induction of TGF-β signalling in specific cells such as DCs and T cells might circumvent these adverse side effects. The results of this study will aid the development of immunotherapies to combat a broad range of inflammatory diseases.

Translational aspects/clinical perspectives

In the present paper, we discovered that TGFβ expressed by DCs plays an important role in the modulation of the immune system during atherosclerosis, and consequently in mediating the progression of atherosclerosis. Dendritic cell-specific TGFβ not only mediates DC polarization, but is even more important in keeping the different T-lymphocytes in control, both within the plaque and in blood and lymphoid organs. This renders TGFβ as one of the most powerful regulators of the immune system in atherosclerosis. Dampening of the immune responses by stimulation of TGFβ would therefore slow down the progression of atherosclerosis and induce atherosclerotic plaques with a low inflammatory burden, thereby reducing the incidence of atherosclerotic plaque rupture and subsequent clinical symptoms such as myocardial infarction or stroke.

Until now, different TGFβ agonists have been developed and their efficacy was tested in numerous animal models. However, besides an immune-modulatory cytokine, TGFβ is also a profibrotic factor, and systemic therapy consequently induces interstitial and liver fibrosis. Based on our present data, stimulation of TGFβ in DCs only would still be beneficial for atherosclerosis by dampening immune responses, but may prevent the adverse effects of global TGFβ stimulation.

Supplementary material

Supplementary material is available at European Heart Journal online.

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References


