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**Title:** Damage associated molecular patterns and toll like receptors in inflammation mediated vascular remodeling : mechanistic insights and therapeutic potentials

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SUPPLEMENTAL MATERIAL TO CHAPTER 2

T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development

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Abstract

Objective T-cells are central to the immune response responsible for native atherosclerosis. The objective of this study is to investigate T-cell contribution to post-interventional accelerated atherosclerosis development, as well as the role of the CD28-CD80/86 co-stimulatory and Cytotoxic T-Lymphocyte Antigen (CTLA)-4 co-inhibitory pathways controlling T-cell activation status in this process.

Methods and Results The role of T-cells and the CD28-CD80/86 co-stimulatory and CTLA-4 co-inhibitory pathways were investigated in a femoral artery cuff mouse model for post-interventional remodeling, with notable intravascular CTLA-4+ T-cell infiltration. Reduced intimal lesions developed in CD4-/- and CD80-/-CD86-/- mice compared to normal C57Bl/6J controls. Systemic abatacept-treatment, a soluble CTLA-4 Ig fusion protein that prevents CD28-CD80/86 co-stimulatory T-cell activation, prevented intimal thickening by 58.5% (p=0.029).

Next, hypercholesterolemic ApoE3*Leiden mice received abatacept-treatment which reduced accelerated atherosclerosis development by 78.1% (p=0.040) and prevented CD4 T-cell activation, indicated by reduced splenic fractions of activated KLRG1+, PD1+, CD69+ and CTLA-4+ T-cells. This correlated with reduced plasma interferon-γ and elevated interleukin-10 levels. The role of CTLA-4 was confirmed using CTLA-4 blocking antibodies, which strongly increased vascular lesion size by 66.7% (p=0.008), compared to isotype-treated controls.

Conclusions T-cell CD28-CD80/86 co-stimulation is vital for post-interventional accelerated atherosclerosis development and is regulated by CTLA-4 co-inhibition, indicating promising clinical potential for prevention of post-interventional remodeling by abatacept.
**Introduction**

Atherosclerosis is a chronic inflammatory disease in which endothelial dysfunction leads to retention of oxidized low-density lipoprotein (oxLDL) cholesterol particles, attracting leukocytes and leading to a local inflammatory response[1-3]. T-cell subsets have been shown to play a vital role in this process[4, 5]. Unlike native atherosclerosis, their contribution to post-interventional remodeling and accelerated atherosclerosis development remains uninvestigated. Although local intimal hyperplasia consist predominately of smooth muscle cells (SMCs) and connective tissue[6], platelet and leukocyte (e.g. T-cells) adherence and activation have been shown to be driving factors behind this overshooting inflammatory healing response, leading to re-occlusion[7].

T-cell responses to immunogenic (neo) antigens such as oxLDL cholesterol are regulated by antigen recognition signals provided by peptide-MHC antigen complexes on antigen-presenting cells (APCs) that bind to the T-cell antigen receptor (TCR), which operates in concert with co-stimulatory signals. The dominant co-stimulatory receptor CD28 is constitutively expressed on resting T-cells, whereas Cytotoxic T-lymphocyte antigen (CTLA)-4 is a co-inhibitory receptor expressed on activated T-cells[8, 9]. Their ligands CD80 and CD86 are upregulated upon activation and predominantly expressed on dendritic cells, B cells, and monocytes/macrophages. CTLA-4 is homologous to CD28 and binds CD80-CD86 with much higher affinity than CD28[10]. During an ongoing immune response, CTLA-4 is upregulated and outcompetes CD28 leading to inhibition of T-cell proliferation and reduction of interleukin (IL)-2 production[11]. The importance of the CD28/CTLA-4 pathways has become evident by generating mice genetically deficient in CTLA-4, which develop fatal lymphoproliferative disease with progressive T-cell accumulation in peripheral lymphoid and solid organs[12, 13].

Upon stimulation, CD4+ T helper 1 (Th1) effector cells upregulate CD40 ligand and produce interferon (IFN)-γ, responsible for pro-atherogenic cellular chemotaxis and macrophage activation [3], leading to inflammation. Blocking of CD28-CD80/CD86 mediated co-stimulation by CTLA-4 domain-containing Ig fusion proteins (abatacept), capable of binding CD80/86 with high-affinity can downregulate T-cell proliferation[14] and production of tumor necrosis factor (TNF)-α, IL-2 and IFN-γ in vitro[15]. Abatacept displays little immunogenicity, with <3% of patients developing an antibody response towards abatacept[16] and is used to treat rheumatoid arthritis (RA) patients[17-19]. Although the T-cell[20, 21] and CD28-CD80/CD86 co-stimulation[5, 22] roles have been demonstrated in native atherosclerosis, their contribution to post-interventional remodeling and accelerated atherosclerosis development is unknown, as is the role of CTLA-4. We hypothesized that the CD28-CD80/86 pathway is instrumental in post-interventional T-cell-regulated arterial inflammation and that the co-inhibitory CTLA-4 pathway downregulates these T-cell responses limiting inflammatory-induced intimal thickening.

In the present study, we studied the role of CD4 T-cells and co-stimulatory cellular activation in post-interventional remodeling in both CD4-/- and CD80-/-CD86-/- mice using a well-established mouse model[7, 23]. CTLA-4 contribution to this process is investigated by treating operated C57Bl/6 mice with abatacept. Next, CTLA-4 co-inhibition effects are investigated by studying post-interventional accelerated atherosclerosis development in hypercholesterolemic ApoE3*Leiden mice after both abatacept treatment and systemic CTLA-4 antibody blockade. Our results demonstrate that CD4 T-cells promote post-interventional atherosclerosis in a CD28-CD80/CD86-dependent fashion and that CD4 T-cell CTLA-4 co-inhibition regulates accelerated atherosclerosis development and bears
high potential for prevention of post-revascularization vascular remodeling.

Methods
The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology[24].

Femoral arterial cuff mouse model
All experiments were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center and the investigations are in conformation with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We performed multiple in vivo studies in which wildtype (C57Bl/6) control, CD4-/- and CD80-/-CD86-/- mice were subjected to femoral artery cuff placement to induce vascular injury and remodeling[7, 23]. Both during surgery and sacrifice, mice were anesthetized with a combination of IP injected Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen). This surgery produces concentric intimal lesions that affect vessel patency and consist predominately of SMCs and connective tissue and is strongly inflammation-dependent[6].

Abatacept treatment
Treatment with 10 mg/kg abatacept at the time of surgery through intraperitoneal injection, similarly to that used in clinical treatment of RA and in earlier murine studies was given to evaluate the role of the CD28-CD80/CD86 co-stimulatory pathway in this process.

Vascular wall lesion analysis
In these vascular segments, inflammatory cell adhesion, infiltration, intimal thickening and lesion composition were assessed using histology, morphometry and immunohistochemistry (IHC), as described previously[23]. Samples were stained with hematoxylin-phloxine-saffron and specific vessel wall composition was visualized for elastin, collagen and with antibodies against leukocytes, macrophages, vascular SMCs, CD3 and CD4 T-cells, matrix metalloproteinase-9 and CTLA-4. This analysis was repeated in operated hypercholesterolemic ApoE3*Leiden mice to assess accelerated atherosclerotic lesion phenotype.

Flow cytometry
Leukocyte subsets were characterized using flow cytometry in spleen and draining inguinal lymph nodes[25]. These were harvested and single-cell suspensions were prepared by mincing the tissue through a 70-µm cell strainer. For cell surface staining, cells were resuspended in staining buffer and incubated with fluorescent conjugated antibodies. After washing and resuspension in staining buffer, cells were acquired using a BD LSRII flow cytometer and data was analyzed using FlowJo software. Cells were stained with fluorochrome-conjugated monoclonal antibodies specific for CD3, CD4, CD44, CD25, CD62L CD69, CD127, CTLA-4, and KLRG1 and staining for intracellular FoxP3 was performed using the FoxP3 staining set. 7-AAD was used to exclude dead cells.

Biochemical analysis
Plasma IFN-γ and IL-10 levels were determined using ELISA were performed according to the manufacturer’s instructions and total plasma cholesterol was measured enzymatically.

Functional CTLA-blockade
CTLA-4 co-inhibition effects on accelerated atherosclerosis development was confirmed using anti-CTLA-4 blocking antibodies[11]. Anti-murine CTLA-4 IgG antibodies used in this study were isolated from supernatants from the 9H10 hybridoma line. Antibody concentration was performed using an artificial kidney and the concentrated antibodies were
protein G-purified. CTLA-4 blockade in ApoE3*Leiden mice was induced by injecting animals IP with 200 μg of anti-mouse CTLA-4 or control IgG once every 2 days, starting at the time of surgery. All materials and methods are described in detail in the supplemental material.

**Results**

CD4 T-cells and CD80/86 mediated-co-stimulation are critically involved in post-interventional vascular remodeling

To investigate the contribution of CD4 T-cells and the CD28/CTLA-4-CD80/CD86 pathways to vascular remodeling, we placed femoral artery cuffs in control, CD4-/- and CD80/-/-CD86/-/- mice and animals receiving abatacept-treatment. Murine body weights were similar at surgery and sacrifice (table 1). 21d after surgery lesions were stained with hematoxylin-phloxine-saffron (HPS) to visualize overall vascular wall morphology (fig 1A), and revealed that untreated animals, compared to CD4-/-, CD80-/-CD86-/- and abatacept-treated mice, developed concentric intimal thickening leading to luminal stenosis. Weigert’s elastin staining was performed to allow morphometric analysis using the elastic laminae to assess vessel layer surface area and remodeling. Analysis showed that intimal thickening was reduced by 72.1% (p=0.006) in CD4-/- mice, by 64.2% (p=0.015) in CD80-/-CD86-/- mice and by 58.5% (p=0.029) in abatacept-treated animals compared to controls (fig 1B).

Since the total surface area (μm2) of the media was similar in all groups (fig 1B), absence of CD4 T-cells and CD80/86 co-stimulatory molecules and abatacept-treatment led to reduced intima / media ratio with respectively 70.0% (p=0.011), 66.4% (p=0.005) and 55.3% (p=0.047, fig 1C). Additionally, the percentage luminal stenosis was reduced in these groups by 48.7% (p=0.042), 47.7% (p=0.031) and 49.9% (p=0.036) respectively (fig 1D). These results indicate a reduced inflammatory-driven remodeling process the injured arterial segments. The total vessel and luminal areas (μm2) were not different between all groups (fig 1A,C). These data indicate an important role of CD4 T-cells and the CD28/CTLA-4-CD80/CD86 co-stimulatory axis in post-interventional vascular remodeling.

![Figure 1](image-url)
Figure 1. Reduced vascular remodeling in CD4−/− and CD80−/−CD86−/− and abatacept-treated mice. Representative cross-sections of cuffed-femoral arteries of CD4−/−CD80−/−CD86−/− and abatacept-treated mice (A) after 21d (hematoxylin-phloxine-saffron (HPS) and Weigert’s elastin staining, 80x, arrows indicate internal elastic laminae). Quantification of intimal thickening (µm²) (B), intima / media ratio (C) and luminal stenosis (%) (D). Representative cross-sections of cuffed-femoral arteries of CD4−/−CD80−/−CD86−/− and abatacept-treated mice (E) after 21d (α-SMC actin and Sirius Red collagen staining, 80x). Quantification of relative medial SMC (F) and collagen (G) areas (%) and intimal SMC (H) and collagen (I) areas (%). Horizontal bars indicate median values, n=10. * p<0.05, ** p<0.01.
CD4-/-, CD80-/-CD86-/- and abatacept-treated mice display an altered lesion composition during post-interventional vascular remodeling. Lesion composition was analyzed using IHC, which showed larger relative areas (to the total vessel layer surface area) of α-SMC actin in the media by 15.8% (p=0.028) in CD4-/- and 16.1% (p=0.028) in abatacept-treated mice (fig 1E), whilst α-SMC actin+ relative area in the intima was significantly decreased compared to controls by 31.6% (p=0.001) and 29.3% (p=0.019) respectively (fig 1G). Both medial (fig 1H) and intimal (fig 1I) relative collagen areas (%) were similar between groups, reflecting absolute α-SMC actin+ and collagen areas (μm2) in the media (fig IIA,C). Total α-actin+ SMC area (μm2) in the intima was only reduced in CD4-/- mice by 59.7% (p=0.008) whilst collagen area (μm2) remained unchanged (fig IIB,D) with limited CD45 leukocyte and CD4 T-cell infiltration in the vascular layers in control sections (fig IIE), indicating an indirect but clear effect of CD4 T-cells upon vascular SMC proliferation and migration.

Abatacept prevents accelerated atherosclerosis
The contribution of the CD28-CD80/CD86 co-stimulatory pathway to accelerated atherosclerosis development was tested in a preclinical model of accelerated atherosclerosis development in Western-type diet-fed hypercholesterolemic ApoE3*Leiden mice using abatacept as therapeutic intervention strategy. Plasma cholesterol concentrations (12.1±3.1 mmol/L) were similar in all groups throughout this study (table 2). Vehicle and abatacept-treated ApoE3*Leiden mice were sacrificed 14d after arterial cuff placement and accelerated atherosclerotic lesions were stained with HPS to visualize overall vascular morphology (fig 2A). This revealed that vehicle-treated animals developed concentric intimal thickening and luminal stenosis, consisting of connective tissue with profound cellular infiltration which was absent in abatacept-treated mice. Quantitative analysis of cuffed arteries stained with Weigert’s elastin identified reduced intimal thickening after abatacept treatment with 78.1% (p=0.040, fig 2B). Abatacept also decreased absolute medial surface area (μm2) by 22.9%
Abatacept prevents accelerated atherosclerosis in hypercholesterolemic mice. Representative cross-sections of cuffed-femoral arteries of hypercholesterolemic ApoE3*Leiden mice following vehicle or abatacept-treatment (A) after 14d (HPS and Weigert's elastin staining, 80x, arrows in inserts indicate internal elastic laminae). Quantification of intimal thickening (µm²) (B), medial area (µm²) (C), intima/media ratio (D) and luminal stenosis (%) (E). Horizontal bars indicate median values, n=10. * p<0.05.

(p=0.040, fig 2C), and intima/media ratio by 69.5% (p=0.037, fig 2D). Furthermore, luminal stenosis percentage was reduced by 48.2% (p=0.021, fig 2E), identifying a potent role for CTLA-4 co-inhibition controlling inflammatory post-interventional vascular remodeling. The total vessel and luminal areas (µm²) were similar in the abatacept-treated group, although a trend towards reduced total vessel area by 34.6% was observed (p=0.094) (fig IIIA, B).

Abatacept positively affects accelerated atherosclerotic lesion composition
Lesion composition was analyzed using IHC to allow arterial wall inflammatory phenotype assessment (fig 3A). Abatacept-treatment produced an altered lesion composition with a reduced inflammatory phenotype. Abatacept reduced leukocyte and macrophage/foam cell fractions (% of all cells) in the media by 64.0% (p=0.043, fig 3B) and 72.1% respectively (p=0.003, fig 3D) and intima by 73.9% (p=0.009, fig 3C) and 30.5% (p=0.048, fig 3E), respectively. Abatacept-treatment also led to a comparable medial (p=0.602, fig 3F) and 29.8% (p=0.042, fig 3G) increased intimal α-SMC actin surface area (%), although absolute intimal SMC area (µm²) was not increased (p=0.743, fig IVA), similarly to the tunica media (p=0.888, fig IVB). Whereas limited CD3 T-cells and matrix metalloproteinase-9 expressing cells could be detected in the tunica intima (fig 3H, I), corresponding with a similar collagen quantity in the intima, abatacept reduced these cells in the tunica adventitia by 65.9% (p<0.0001, fig 3J) and 64.7% (p<0.0001, fig 3K) respectively. T-cell CTLA-4 and CD4 co-localized expression was identified using IHC in the injured arterial segments of vehicle-treated ApoE3*Leiden mice and was absent in uninjured arteries, but occurred 3d after surgery and could still be observed 14d after injury, indicating continuing local arterial T-cell activation throughout the remodeling process and accelerated atherosclerosis development (fig 3L).
Figure 3 (next page). Abatacept positively affects accelerated atherosclerotic lesion composition.

Representative cross-sections of cuffed-femoral arteries of ApoE3*Leiden mice following vehicle or abatacept-treatment (A) after 14d (leukocyte, macrophage and α-SMC actin staining, 80x). Quantification of relative medial leukocyte (B), macrophage (D) and SMC (F) areas (%) and intimal leukocyte (C), macrophage (E) and SMC (G) areas (%) and intimal CD3 (n) (H) and MMP-9 (n) (I), as well as adventitial CD3 (n) (J) and MMP-9 cells (n) (K). Horizontal bars indicate median values, n=10. * p<0.05, ** p<0.01. (L) T-cell CTLA-4 and CD4 expression throughout the vessel wall of ApoE3*Leiden mice in time before and 3d and 14d after surgery (CTLA-4 and CD4 staining, 80x, arrows in inserts indicate positive staining).
Abatacept prevents systemic CD4 T-cell activation during accelerated atherosclerosis

To examine the role of CTLA-4 co-inhibition upon T-cell activation involved in accelerated atherosclerosis development, CD4 T-cell numbers and T-cell activation status were assessed. The markers Killer cell lectin-like receptor subfamily G member (KLRG)-1, Programmed Death (PD)-1, CD69 and CTLA-4 were used to analyze T-cell activation in the splenic reservoir and draining inguinal lymph nodes of mice 14d after surgery.

Absolute CD4 (p=0.557, fig 4A) splenic T-cell numbers were similar in vehicle and abatacept-treated mice, as were total splenic cell contents (absolute cells) and percentages CD4 T-cell fractions (fig VA,B). Abatacept reduced splenic CD4 T-cells fractions expressing CD69 by 61.9% (p=0.008, fig 4B), PD1 by 49.4% (p=0.041, fig 4C), KLRG1 by 47.4% (p=0.032, fig 4E), and CTLA-4 by 47.0% (p=0.016, fig 4F). These data indicate that abatacept strongly and consistently prevented systemic CD4 T-cell activation, thereby reducing accelerated atherosclerotic lesion formation. In contrast to the reduced severity of inflammatory vascular remodeling, co-inhibition with abatacept treatment also reduced fractions of CD4+CD25+FoxP3+ regulatory T-cells by 33.3% (p=0.016, fig 4H) in the spleen. No significant differences in the percentages of naive (CD62L+ CD44-), central-
memory (CD62L+ CD44+) or effector-memory (CD62L- CD44+) CD4 T-cell populations were observed (p>0.05, fig VC-F).

Contrary to significant reduction of activated CD4 T-cells in the spleen after abatacept-treatment, no evidence of T-cell activation in draining inguinal lymph nodes could be found (fig VIA-G), despite adequate cell number isolation for analysis.

Figure 4. Abatacept prevents systemic CD4 T-cell activation in ApoE3*Leiden mice. Abatacept did not affect total CD4 splenic T-cell count (absolute cells) 14d after surgery (A). T-cells were analyzed using multiparametric flow cytometry and gated for 7AAD-, CD3+ and CD4+ markers and subsequently for either KLRG1+, PD1+, CD69+, CTLA-4+ or CD25+ and FoxP3+ expression and are displayed in dot plots (D and G). Abatacept reduced CD69+ (B), PD1+ (C), KLRG1+ (E) and CTLA-4+ (F) activated CD4 T-cell fractions (%), together with CD25+FoxP3+ regulatory (H) CD4 T-cell fractions (%). Horizontal bars indicate median values, n=5. * p<0.05, ** p<0.01.
**Abatacept affects systemic cytokine levels during accelerated atherosclerosis**

Contribution of activated T-cell fractions to vascular remodeling severity is supported by the positive correlation ($R^2=0.61$, $p=0.002$) between KLRG1+ CD4+ T-cells (%) and intimal thickening ($\mu$m2) (fig 5A). Interestingly, the ratio between regulatory and effector helper T-cells remained unchanged in treated animals (fig 5B). Affected systemic CD4 T-cell activation in abatacept-treated mice was investigated by IFN-γ and IL-10 measurements in plasma at 14d. Hypercholesterolemic control animals contained an elevated plasma concentration IFN-γ of $43.2\pm12.1$ pg/ml, while abatacept-treatment reduced the IFN-γ concentration to non-detectable levels ($p=0.00023$, fig 5C) after vascular injury. In contrast, plasma IL-10 was significantly elevated from $4.5\pm2.0$ pg/ml in controls to $23.7\pm7.1$ pg/ml ($p=0.012$, fig 5D) in abatacept-treated mice. Together, these cytokines indicate that abatacept reduced systemic inflammation.

**CTLA-4 blockade exacerbates accelerated atherosclerosis development.**

To confirm the anti-inflammatory role of CTLA-4 co-inhibition in accelerated atherosclerotic lesion development, we analyzed injured femoral lesions of mice undergoing CTLA-4 blockade using antibodies. HPS staining (fig 6A) revealed that CTLA-4 blocking provoked increased accelerated atherosclerosis development and decreased luminal patency. Weigert’s elastin staining and morphometric vessel wall analysis confirmed increased intimal thickening by 66.7% compared to control mice receiving non-specific anti-β gal IgG ($p=0.008$, fig 6B). Due to a comparable medial area between groups, the intima / media ratio was increased by 69.3% ($p=0.010$, fig 6C). Finally, CTLA-4 blockade increased relative luminal stenosis by 86.3% ($p=0.004$, fig 6D) and compromised absolute luminal area by 56.9% ($p=0.010$, fig 6E).
Total vessel and medial areas (μm²) were both not different between groups (fig VIIA,B). Together, these results indicate a strong increase of inflammatory induced post-interventional vascular remodeling during functional blockade of CTLA-4 function.

**Accelerated atherosclerotic lesion phenotype is preserved during CTLA-4 blockade**

Lesion composition was analyzed using IHC to assess arterial wall inflammatory phenotype (fig VIIIA). Although CTLA-4 blockade enhanced accelerated atherosclerosis development, no lesion composition differences were observed compared to controls. There were similar leukocyte and macrophage/foam cell fractions (% of all cells) in the media (p=0.414, fig VIIIB; p=0.097, fig VIIID, respectively) and intima (p=0.142, fig VIIIC; p=0.769, fig VIIIE, respectively). CTLA-4 blockade also led to a comparable medial (p=0.728, fig VIIIF) and intimal (p=0.258, fig VIIIG) α-SMC actin surface areas (%). IHC revealed that functional CTLA-4 blockade led to a significant increase of CD3+ T-cells (n) in both the tunica intima by 200.0% (p=0.0001, fig 6F) and in the tunica adventitia by 241.4% (p=0.0003, fig 6G) compared to controls.

**Figure 6.** CTLA-4 blockade exacerbates accelerated atherosclerosis development. Representative cross-sections of cuffed-femoral arteries of hypercholesterolemic ApoE3*Leiden mice following isotype antibody or anti-CTLA-4 IgG-treatment (A) after 14d (HPS, Weigert’s elastin and CD3 staining, 80-160x, arrows in inserts indicate internal elastic laminae and CD3+ T-cells). Quantification of intimal thickening (μm²) (B), intima / media ratio (C), luminal stenosis (%) (D) and luminal area (μm²) (E). Quantification of intimal CD3 (n) (F) and adventitial CD3 (n) (G) cells. Horizontal bars indicate median values, n=10. *p<0.05, **p<0.01.
Discussion

This study demonstrates an important role for T-cell co-stimulation by CD28-CD80/CD86 and the negative regulator CTLA-4 in post-interventional intimal hyperplasia and accelerated atherosclerosis development. We show that CD4-/- and CD80-/-CD86-/- mice develop significantly smaller SMC-rich lesions following vascular injury and that infiltrated T-cells express CTLA-4 early after surgery. Therapeutic inhibition of CD28-CD80/86 function by abatacept significantly prevented accelerated atherosclerosis development in hypercholesterolemic ApoE3*Leiden mice together with reduced IFN-γ plasma concentration (fig 5C), probably due to reduced CD4 T-cell activation. The role of CTLA-4 co-inhibition was confirmed using blocking antibodies which led to profound increased vascular lesion size.

The role of CD4 T-cells and CD80/86-mediated co-stimulation during native atherosclerosis development[26, 27] has been investigated, but their contribution to post-interventional vascular remodeling remained until this study unknown. In ApoE-/- mice, T-cells have been shown to be important in early native atherosclerotic lesion progression, but not its initiation[27, 28]. We provide evidence for activated CD4 T-cell involvement in the early stage of vascular remodeling following intervention. As of yet, it remains to be investigated towards which antigen the T-cell response is raised. Nevertheless, this form of accelerated atherosclerosis develops within months and in a localized area, opening the perspective for (local) T-cell-directed treatment directly after percutaneous coronary interventions.

CTLA-4 is expressed on activated effector T-cells and constitutively on regulatory T-cells, while both CD80 and CD86 are primarily expressed on activated dendritic cells, B cells, monocytes/macrophages, which play an important role in native atherosclerosis development [4]. T-cell-mediated immune responses are initiated in lymphoid tissues where they are activated by APCs presenting two concomitant signals: an antigenic signal provoked by specific MHC-antigen peptide complexes that bind to the TCR and a co-stimulatory signal induced by CD80 and CD86 molecules that interact with CD28 expressed on all T-cells. Together, TCR and CD28 signals lead to an inflammatory and pro-atherogenic response.

We show that abatacept-treatment was able to prevent this systemic activation by reducing the number of activated CD4 T-cells fractions in the spleen as evidenced by the activation markers KLRG1, PD1, CD69 and CTLA-4. Since vascular injury occurs locally, profound T-cell activation was expected to occur in draining lymph nodes. However, only low fractions of activated T-cells were identified in these lymph nodes (fig VIA-G) whilst analysis of splenic T-cell fractions revealed significant activation (fig 4A-F). We cannot exclude effects of abatacept in other lymph nodes, but these are unlikely to supersede the effects in the analyzed primary draining lymph nodes.

Although abatacept effects on accelerated atherosclerosis are mediated through systemically reduced T-cell activation, it is not clear to which extent effects on specific T-cell subsets contributed to the lesion development. Buono et al.[22] showed that CD80-/-CD86-/-LDL-receptor (r)-/- mice developed decreased atherosclerotic lesions compared to LDL-r-/- mice through reduced CD4 Th1 cell activation by CD80/86 on APCs. Unlike activated effector T-cells, regulatory CD25+ FoxP3+ T-cells constitutively express CTLA-4[29] and this is essential for their suppressive function[30, 31]. Ait-Oufella et al.[5] demonstrated the vital role of regulatory T-cells in native atherosclerosis development , using irradiated LDL-r-/- mice receiving reconstituted bone-marrow from CD80-/-CD86-/- mice which developed increased atherosclerotic lesions through strongly reduced splenic fractions of regulatory CD25+FoxP3+ T-cells. Therefore, it is possible that abatacept, may have inhibitory effects
on accelerated atherosclerosis development through regulatory T-cell activation status modulation. However, Platt et al. showed that abatacept prevented effector T-cell activation, although effects on regulatory T-cell status in this murine arthritis model were not studied[14].

This study shows reduced fractions of both activated Th1 and regulatory T-cells in hypercholesterolemic ApoE3*Leiden mice receiving abatacept. Since the Th1: regulatory T-cell ratio remained unchanged (fig 5B), this strongly suggests that therapeutic effects of abatacept can primarily be attributed to directly reduced activation of Th1 T-cells and not to Th1 suppression through regulatory T-cells, reflected by an elevated concentration of IL-10 (fig 5D) in plasma (baseline 14.4±2.3 pg/ml)[23] and reduced concentration of IFN-γ (fig 5C) (baseline 9.8±4.6 pg/ml)[23], similarly to that observed following IL-12 vaccination by Hauer et al. which downregulated the Th1 immune response and led to attenuation of native atherosclerosis development[32].

These findings are based upon results from animal studies and cannot be automatically extrapolated to the clinical situation. However, they do provide further insight into the role of T-cell co-stimulatory pathways when viewed in the light of recent exiting data by Dumitriu et al.[33] concerning the role of CD28/-/- activated T-cells and regulatory T-cells during acute coronary syndromes (ACS) in humans, as well as carotid stenosis[34], unstable angina[35, 36] and atherosclerosis development in both humans[37] and animals in general[38]. The function relevance of CTLA-4 expression for human peripheral T-cell has previously been shown by Hoff et al.[39] and Yi-qun et al.[40], in which CTLA-4 blockade was found to both determine CD28null T-cell and memory T-cell longevity and responsiveness (e.g. IL-2 production). The potential of co-stimulatory-based T-cell-directed intervention strategies in the clinical situation is further enhanced by the findings of Bluestone et al.[41] and Körmendy et al.[42], in which functional CTLA-4 blockade functionally affected circulating regulatory T-cells in patients undergoing kidney transplantation and peripheral Th cells in rheumatoid arthritis patients. This study demonstrated the effectiveness of modulation of co-stimulatory receptors against post-interventional atherosclerotic vascular remodeling in mice, thus greatly enhancing the potential of a similar therapeutic approach to improve the survival of ACS patients.

In conclusion, this study shows that T-cell co-stimulation through the CD28-CD80/86 pathway plays a vital role in post-interventional accelerated atherosclerosis development and is strongly negatively regulated by CTLA-4 co-inhibition. These results may have important clinical implications. Immune-mediated interventions directed towards therapeutically controlling the inflammatory T-cell response such as abatacept are widely applied in other immune (e.g. rheumatoid) disorders and could now be used in an early phase following interventions such as revascularization or bypass grafting procedures in patients to prevent subsequent vascular remodeling. This application could be accelerated by the availability of abatacept as a currently clinically approved T-cell specific therapeutic agent.

Disclosures None.

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Online supplements
T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development

Materials and Methods

Mice
All experiments were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC). Male C57Bl/6J controls, CD4-/- and CD80-/CD86-/- mice on a C57Bl/6J background were purchased from the Jackson Laboratory (Bar Harbor) and transgenic male ApoE*3-Leiden mice, backcrossed for more than 20 generations on a C57BL/6J background, were bred in our own laboratory. All animals used for this experiment were 10-12 weeks at the start of a dietary run-in period or surgery and were weighed before and at the end of the experimental period.

Diets
C57Bl/6J controls, CD4-/- and CD80-/CD86-/- mice received chow diet and transgenic male ApoE*3-Leiden mice were fed a Western-type diet containing 1% cholesterol and 0.05% cholate to induce hypercholesterolemia to desired levels in male ApoE3*Leiden mice (AB Diets). The Western-type diet was given three weeks prior to surgery and was continued throughout the experiment. All animals received food and water ad libitum during the entire experiment.

Treatment protocol
To investigate the role of CTLA-4 co-inhibition, C57Bl/6J control and ApoE3*Leiden animals were injected intraperitoneally (IP) with abatacept (Bristol-Myers Squibb B.V.) in a concentration of 10 mg/kg twice monthly (200 μl) or vehicle, starting at the time of surgery. CTLA-4 blockade in ApoE3*Leiden mice was induced by injecting animals IP with 200 μg of anti-mouse CTLA-4 IgG (clone 9H10[1]) or control IgG diluted in 200 μl sterile phosphate-buffered saline (PBS) once every 2 days, starting at the time of surgery.
Blocking CTLA-4 IgG antibody generation

Anti-murine CTLA-4 IgG antibodies used in this study were isolated from supernatants from the 9H10 hybridoma line[1], first using Iscove’s Modified Dulbecco’s Medium (IMDM) (Invitrogen) with 8% FCS and 1% glutamine, followed by GIBCO™ protein-free hybridoma medium (PFHM)-II (Invitrogen) throughout T75, T175 and roller bottle culture systems (Sigma-Aldrich Chemie B.V), maintained at 37°C with 5% CO2. Antibody concentration was performed using an artificial kidney (Fresenius Medical Care). The concentrated antibodies were protein G-purified (GE Healthcare Bio-Sciences AB) and antibody concentrations were determined using a Nanodrop spectrophotometer and stored at 2 mg/ml in sterile PBS at -20°C for further use.

Femoral artery cuff mouse model

To investigate the role of CD4 T lymphocytes, CD28-CD80/CD86 co-stimulation and CTLA-4 co-inhibition, mice were subjected to arterial femoral arterial cuff placement to induce intimal thickening and accelerated atherosclerosis development[2-4]. In brief, mice were anesthetized before surgery with a combination of IP injected Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen). The right femoral artery was isolated and sheathed with a rigid non-constrictive polyethylene cuff (Portex, 0.40mm inner diameter, 0.80mm outer diameter and an approximate length of 2.0mm). Using two ligatures, the polyethylene cuff was held in place, after which the wound was closed using continuous sutures.

Either 14 days (ApoE3*Leiden mice) or 21 days (normcholesterolemic mice) after cuff placement, mice were anesthetized before surgery and euthanized. At sacrifice, venous blood was drawn in EDTA collection tubes (Sarstedt B.V) and subjected to centrifugation (6000 r.p.m. for 10 min at 4°C) to obtain plasma, which was stored at -20°C for further research. The spleen and draining inguinal lymph nodes were isolated and either snap frozen in liquid nitrogen for further analysis (stored at -80°C) or minced through a 70-mm cell strainer (BD Biosciences) to create single-cell suspensions and stored in 3% fetal calf serum-rich GIBCO™ RMPI 1640 medium (Invitrogen) on ice.

Next, the thorax was opened and mild pressure-perfusion (100mm Hg) with PBS for 4min by cardiac puncture in the left ventricle. After perfusion, the cuffed femoral artery was harvested, fixed overnight in 3.7% formaldehyde in water (w/v) and paraffin-embedded. Serial cross-sections (5 μm thick) were made from the entire length of the artery for analysis.

Biochemical analysis

Total plasma cholesterol (Roche Diagnostics, kit 1489437) concentration was measured enzymatically before randomization and at sacrifice. Plasma cholesterol concentrations (12.1±3.1 mmol/L) were similar in all groups throughout this study (p>0.15, table 2). To investigate effects of abatacept on systemic CD4 T-cell specific activation after surgery, interferon (IFN)-γ and interleukin-10 enzyme-linked immuno sorbent assays (ELISAs) were performed (BD Biosciences) according to the manufacturer’s instructions.

Quantification of cuffed femoral artery lesions

Immunohistochemical (IHC) staining was performed using positive and negative tissue-specific controls as indicated by the antibody manufacturer. Samples were stained with hematoxylin-phloxine-saffron (HPS) and specific vessel wall composition was visualized.
for elastin (Weigert’s elastin staining), collagen (Sirius Red staining) and with antibodies against leukocytes (anti-CD45 antibodies 1:200, Pharmingen), macrophages (MAC3, 1:200, BD Biosciences), vascular SMCs (α-SMC actin 1:800, Dako), T-cells (anti-CD3, 1:100, AbD Serotec and anti-CD4, 1:250, Abcam), matrix metalloproteinase-9 (anti-MMP-9, 1:100, Santa Cruz), and CTLA-4 (anti-mouse CTLA-4, 1:200, Abbiotec), using hematoxylin for counterstaining to visualize all cells. The anti-CD4 antibody specificity for CD4 was confirmed on cuffed sections of CD4 knock-out mice (21d) (fig IIF).

Sections were deparaffinized by placement in xylene for 5 minutes, followed by ethanol 100% for 2 minutes, ethanol 70% for 2 minutes and ethanol 50% for 2 minutes and distilled water for 2 minutes. Sections underwent citrate buffer antigen retrieval (10mM sodium citrate, pH 6.0) for 10 minutes at 100°C, followed by 30 minutes cooling. PBS (1%) was used as a wash solution and for diluting antibodies. Novared (Vector laboratories) was used as staining vector according to the manufacturer’s instructions. Sections were dehydrated by placement in ethanol 50% for 2 minutes, ethanol 70% for 2 minutes and ethanol 100% for 2 minutes, followed by xylene for 5 minutes. The slides were mounted with xylene-based pertex and 24x60 mm coverslips.

The number of leukocytes, macrophages and cells expressing CTLA-4 attached to the endothelium, within the neointimal tissue or infiltrated in the medial layer of the femoral arteries was quantified and is displayed as a percentage of the total number of present cells. The area containing vascular SMCs, collagen or macrophages was quantified using computer-assisted morphometric analysis (Qwin, Leica) and is expressed as a percentage of the total cross-sectional arterial wall layer area. All quantification in this study was performed on six equally spaced (150µm distance) serial stained perpendicular cross-sections throughout the entire length of the vessel and was performed by blinded observers.

**Flow cytometry**

Spleens and draining inguinal lymph nodes were harvested and single-cell suspensions were prepared by mincing the tissue through a 70-µm cell strainer (BD Biosciences). Erythrocytes were lysed using hypotonic (0.82%) ammonium chloride buffer. For cell surface staining, cells were resuspended in staining buffer (PBS + 3%P PFCS + 0.05% sodium azide) and incubated with fluorescent conjugated antibodies at 4°C for 30 minutes in 96-well plates. After washing and resuspension in staining buffer, cells were acquired using a BD LSRII flow cytometer and data was analyzed using FlowJo software (version 7.4.6., Tree Star).

Cells were stained with fluorescent-conjugated monoclonal antibodies specific for CD3, CD4, CD44, CD25, CD62L CD69, CD127, CTLA-4, and KLRG1. All antibodies were purchased from eBioscience or BD Biosciences. Staining for intracellular FoxP3 was performed using the FoxP3 staining set from eBioscience (1:200, APC). 7-AAD was used to exclude dead cells.

**Statistical analysis**

All data are presented as mean± standard error of the mean (SEM). Groups were compared using a Mann-Whitney sum test for non-parametric data. All statistical analyses were performed with SPSS 17.0 software for Windows or using Prism software. P-values <0.05 were regarded as statistically significant and are indicated with an asterisk (*).
Body weight (gram) of control, CD4−/−, CD80−/−CD86−/− and abatacept-treated (10 mg/kg/twice monthly) C57Bl/6J mice at surgery and sacrifice at day 21 (mean±SEM, n=10). No significant differences were observed.

| Group          | Body weight (gram) | Total cholesterol (mmol/L) |
|----------------|--------------------|-----------------------------|
|                | Surgery            | Sacrifice                  | Surgery | Sacrifice               |
| C57Bl/6J       | 22.6±0.4           | 24.8±0.4                   |         |                         |
| CD4−/−         | 26.7±0.4           | 29.0±0.4                   |         |                         |
| CD80−/−CD86−/− | 24.1±1.1           | 26.0±1.0                   |         |                         |
| Abatacept      | 23.7±0.4           | 25.9±0.3                   |         |                         |

Body weight (gram) of vehicle, abatacept (10 mg/kg/twice monthly), isotype control antibody (anti-β gal IgG 200 µg every 2 days) or anti-mouse CTLA-4 IgG (200 µg every 2 days)-treated ApoE3*Leiden mice measured at surgery and sacrifice (14 days) (mean±SEM, n=10). No significant differences were observed.

| Group                        | Body weight (gram) | Total cholesterol (mmol/L) |
|------------------------------|--------------------|-----------------------------|
|                              | Surgery            | Sacrifice                  | Surgery | Sacrifice               |
| Vehicle                      | 29.0±0.6           | 11.3±0.4                   | 7.9±0.6 |
| Abatacept                    | 29.4±0.7           | 11.5±1.0                   | 10.4±2.4|
| Anti-β gal IgG               | 28.8±0.6           | 13.4±1.4                   | 8.9±0.9 |
| Anti-CTLA-4 IgG              | 30.4±0.7           | 12.3±0.9                   | 8.1±1.5 |

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Figure 1

Figure I. Reduced vascular remodeling in CD4−/− and CD80−/−CD86−/− and abatacept-treated mice. Quantification of total vessel area (µm²) (A), medial area (µm²) (B) and luminal area (µm²) (C) after 21 days. Horizontal bars indicate median values, n=10.

Figure I. T-cell co-stimulation by CD28-CD80/86 and its negative regulator CTLA-4 strongly influence accelerated atherosclerosis development.

Supplemental figures

Luminal area (µm²)

Vehicle CD4−/− CD80−/− CD86−/− Abatacept

Medial area (µm²)

Vehicle CD4−/− CD80−/− CD86−/− Abatacept

Total vessel area (µm²)

Vehicle CD4−/− CD80−/− CD86−/− Abatacept

222
Figure II. CD4−/−, CD80−/−CD86−/− and abatacept-treated mice display an altered lesion composition. Quantification of medial (A) and intimal (B) vascular α-SMC actin area (µm²) and medial (C) and intimal (D) collagen area (µm²) after 21 days. Representative cross-sections of cuffed-femoral arteries of vehicle-treated C57Bl/6J mice (E) after 21d (CD45 leukocyte and CD4 T-cell staining, 80-160x). (F) Representative cross-sections of cuffed-femoral arteries of CD4 knock-out mice after 21d (HPS and CD4 staining, 80x). Horizontal bars indicate median values, n=10. ** p<0.01.
Figure III. Abatacept prevents accelerated atherosclerosis in hypercholesterolemic ApoE3*Leiden mice. Quantification of total vessel area (μm²) (A) and luminal area (μm²) (B) after day 14. Horizontal bars indicate median values, n=10.

Figure IV. Abatacept positively affects accelerated atherosclerotic lesion composition. Quantification of total intimal (A) and medial (B) SMC areas (μm²) after day 14. Horizontal bars indicate median values, n=10. * p<0.05, ** p<0.01.
Figure V. Abatacept prevents systemic CD4 T-cell activation in ApoE3*Leiden mice. Quantification of splenic total cell count (*10^6 cells) (A), CD4 T-cell fractions (%) (B), splenic naïve (CD62L+CD44-) (C) and activated central-memory (CD62L+CD44+) (D) and effector-memory (CD62L-CD44+) (E) CD3+CD4+ T-cells (%) 14 days after surgery. Horizontal bars indicate median values, n=5.
Figure VI. Abatacept does not affect draining inguinal lymph node T-cell activation status in hypercholesterolemic ApoE3*Leiden mice. Quantification of total cell count (*10⁶ cells) (A), total CD4 T-cells (*10⁶ cells) (B), CD4 T-cell fraction (%) (C), regulatory CD4+CD25+FoxP3+ T-cells (%) (D), activated PD1+ (E), CD69+ (F) and CTLA-4+ (G) CD4 T-cells (%) in draining inguinal lymph nodes, 14 days after surgery. Horizontal bars indicate median values, n=5.
Figure VII. CTLA-4 blockade exacerbates accelerated atherosclerosis development in hypercholesterolemic ApoE3*Leiden mice. Quantification of total vessel area (µm²) (A) and medial area (µm²) (B), 14 days after surgery. Horizontal bars indicate median values, n=10.
Figure VIII

Accelerated atherosclerotic lesion phenotype is preserved during CTLA-4 blockade. Representative cross-sections of cuffed-femoral arteries of ApoE3*Leiden mice following isotype antibody or anti-CTLA-4 IgG-treatment (A) after 14d (leukocyte, macrophage and α-SMC actin staining, 80x). Quantification of relative medial leukocyte (B), macrophage (D) and SMC (F) areas (%) and intimal leukocyte (C), macrophage (E) and SMC (G) areas (%) showed no significant differences. Horizontal bars indicate median values, n=10.
Supplemental material to Chapter 2

Annexin A5 prevents post-interventional accelerated atherosclerosis development in a dose-dependent fashion.

Ewing MM, Karper JC, Sampietro ML, de Vries MR, Pettersson K, Jukema JW, Quax PH

Atherosclerosis 2012;221:333-340.
Abstract

Background Activated cells in atherosclerotic lesions expose phosphatidylserine (PS) on their surface. Annexin A5 (AnxA5) binds to PS and is used for imaging atherosclerotic lesions. Recently, AnxA5 was shown to inhibit vascular inflammatory processes after vein grafting. Here, we report a therapeutic role for AnxA5 in post-interventional vascular remodeling in a mouse model mimicking percutaneous coronary intervention (PCI).

Methods and Results Associations between the rs4833229 (OR=1.29 (CI 95%), p_{allelic}=0.011) and rs6830321 (OR=1.35 (CI 95%), p_{allelic}=0.003) SNPs in the AnxA5 gene and increased restenosis-risk in patients undergoing PCI were found in the GENDER study. To evaluate AnxA5 effects on post-interventional vascular remodeling and accelerated atherosclerosis development in vivo, hypercholesterolemic ApoE^{-/-} mice underwent femoral arterial cuff placement to induce intimal thickening. Dose-dependent effects were investigated after 3 days (effects on inflammation and leukocyte recruitment) or 14 days (effects on remodeling) after cuff placement. Systemically administered AnxA5 in doses of 0.1, 0.3 and 1.0 mg/kg compared to vehicle reduced early leukocyte and macrophage adherence up to 48.3% (p=0.001) and diminished atherosclerosis development by 71.2% (p=0.012) with a reduction in macrophage/foam cell presence. Moreover, it reduced the expression of the endoplasmic reticulum stress marker GRP78/BiP, indicating lower inflammatory activity of the cells present.

Conclusions AnxA5 SNPs could serve as markers for restenosis after PCI and AnxA5 therapeutically prevents vascular remodeling in a dose-dependent fashion, together indicating clinical potential for AnxA5 against post-interventional remodeling.

Keywords
Atherosclerosis, restenosis, inflammation, annexin A5, genetics
Introduction
Post-interventional vascular remodeling and accelerated atherosclerosis development are important complications of revascularization strategies and limit treatment success rate (1). These features are elicited by endothelial and atherosclerotic plaque injury, triggering inflammatory activation and leukocyte recruitment to the injured arterial segment. These cells are the driving factors behind smooth muscle cell (SMC) proliferation and extracellular matrix deposition leading to intimal hyperplasia. Subendothelial retention and oxidation of low-density lipoprotein (LDL) cholesterol is central to the initial lesion formation in both native atherosclerosis and restenosis development (2,3). Recently, it was postulated that endoplasmic reticulum (ER) stress, leading to the unfolded protein response (UPR), is involved in the regulation of inflammation in activated vascular cells and the link between UPR and arterial inflammation is emerging as an important factor in (accelerated) atherosclerosis development (4-7).

AnxA5 is a member of the annexin family of proteins that calcium-dependently bind to negatively-charged phospholipid surfaces and was originally discovered as an anticoagulant and antithrombotic protein (8-11) and has been shown to inhibit the prothrombinase complex (12) and to down-regulate the surface expression of tissue factor (13). It is now known to have anti-inflammatory and anti-atherosclerotic properties (14,15) and to regulate interferon γ signalling (16). Viable cells express phosphatidylserine (PS) on their inner cellular membrane leaflet. When PS is externalized, it serves as an ‘eat-me’ signal. Annexin A5 (AnxA5) binds reversibly, specifically and with high affinity to PS (15). PS becomes externalized during apoptosis, which makes AnxA5 a powerful tool to detect apoptosis (and atherosclerosis) both in vitro and in vivo (17). PS is expressed in native atherosclerosis and after revascularisation procedures, and circulating AnxA5 binds with high affinity to these cells, and is therefore present in high concentrations in atherosclerotic plaques and injured vascular segments. PS externalization is normally thought to be associated to apoptosis, but can also be externalized in a controlled and reversible way in non-apoptotic cells (18,19).

Plasma levels of AnxA5 are inversely related to the severity of coronary stenosis and are indicative of the extent of atherosclerotic plaques (20), but are also elevated in subjects with left ventricular hypertrophy and following myocardial infarction (21,22). It was recently shown that systemically administered AnxA5 can prevent vein graft disease and vascular inflammation (23) and that the dimer of annexin A5, diannexin, can protect against renal ischemia-reperfusion injury and inflammatory cell infiltration into transplanted islet grafts (24,25). Patients with hypercholesterolemia and previous coronary heart disease (CHD) undergoing PCI for atherosclerosis are most at risk for inflammatory-driven post-interventional restenosis development. The risk for development of restenosis may partially be determined by genetic factors. It has been shown that genetic variations in genes encoding inflammatory factors (SNPs) can predict the risk for restenosis after percutaneous coronary intervention (PCI) (26). The effects of genetic variation in the AnxA5 gene on clinical restenosis after PCI or cardiovascular disease progression have thus far not been elucidated.

In the present study we investigated the association between AnxA5 SNPs and restenosis-risk in patients undergoing PCI, followed by in vivo evaluation of the therapeutic effectiveness of AnxA5 in a humanized mouse model for post-interventional vascular remodeling using ApoE3*Leiden mice. Our findings point to a potential diagnostic and therapeutic clinical role for AnxA5 against post-PCI vascular remodeling.
Materials and Methods

Association between single nucleotide polymorphisms (SNPs) in the AnxA5 gene, extracted from the GENDER genome wide association study (GWAS) dataset (27) and restenosis-risk following PCI was investigated.

We performed in vivo intervention studies in which hypercholesterolemic ApoE⁻/⁻ mice on a Western-type diet were subjected to femoral artery cuff placement to induce vascular injury and remodeling (28). Cuff placement leads to a localized vascular inflammation, which in turn produces concentric intimal lesions that can affect vessel patency. The lesions consist of SMCs, connective tissue and infiltrated leukocytes such as macrophages / foam cells and are strongly inflammation-dependent (29). In these vascular segments, inflammatory cell adhesion, infiltration, intimal thickening and lesion composition were assessed using histology, morphometry and immunohistochemistry (IHC), as described previously (29). Treatment with vehicle, 0.1, 0.3 and 1.0 mg/kg AnxA5 was given to operated ApoE⁻/⁻ mice. A three day protocol was used to evaluate effects on leukocyte recruitment, and a 14 day protocol to evaluate effects on vascular remodeling. All materials and methods are described in detail in the supplemental material.

Results

Annexin A5 SNP as risk marker for clinical restenosis

AnxA5 plasma levels are linked to the severity of coronary stenosis and AnxA5 is a marker of cardiovascular disease progress. These data indicate a potential role of AnxA5 in (post-interventional) accelerated atherosclerosis development. Therefore we investigated the association between AnxA5 SNPs and restenosis risk in patients undergoing PCI enrolled in the GENDER study, composed of 866 patients (295 cases that developed restenosis following PCI and 571 controls that did not develop restenosis). Clinical outcome was linked to genetic data obtained through a genome-wide association analysis.

The allelic association test identified two SNPs, rs4833229 and rs6830321, which are significantly associated with restenosis risk after PCI (fig 1A). Both SNPs increased the risk for restenosis (rs4833229, odds ratio (OR) =1.29, (95% confidence interval (CI) 1.06-1.58), \( p_{\text{allelic}} =0.011 \) and rs6830321, OR=1.35 (95% CI 1.10-1.64), \( p_{\text{allelic}} =0.003 \), even after adjustment for clinical risk factors, such as total occlusion, diabetes, smoking and residual stenosis (table 1). The minor allele frequencies for cases and controls from the GENDER population are 0.481 and 0.418 for rs4833229 and 0.510 and 0.436 for rs6830321 respectively, indicating they are present in a large proportion of the population. The AnxA5 gene linkage disequilibrium (LD) plot shows that rs4833229 and rs6830321 are in high LD (\( r^2=0.91 \), fig 1B). Haplotype analysis in the gene showed similar association results with restenosis as found in the single SNP analysis (haplotype ACAGTTGTT, frequency: 0.427, OR=1.275, \( p=0.018 \)). These data link AnxA5 SNPs to restenosis-risk after PCI and suggest that AnxA5 genotype functions as risk marker for restenosis. We therefore further explored AnxA5’s therapeutic potential using an in vivo model for restenosis and intimal hyperplasia.
Annexin A5 dose-dependently prevents leukocyte recruitment after vascular injury

Effects of AnxA5 on leukocyte recruitment to injured arterial segments was investigated in the femoral artery cuff model in ApoE knockout mice receiving daily vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 through IP injection. Total plasma cholesterol was not affected by annexin A5.
treatment (supplementary table I). Three days after cuff placement there is inflammation in the cuffed arteries, with leukocytes both adherent to the endothelial surface and with cells that have migrated into the media layer (fig 2A). Staining of arterial lesions at this time point revealed that 0.1, 0.3 and 1.0 mg/kg/d AnxA5-treated animals displayed a reduced percentage of endothelial leukocyte adhesion by 26.7% (p=0.014), 34.9% (p=0.010) and 48.3% (p=0.001) respectively (fig 2B). For monocytes/macrophages, this percentage was reduced by 40.0% (p=0.029), 66.9%, (p=0.001) and 45.0% (p=0.037) respectively (fig 2C). The percentage leukocyte infiltration into the media was reduced by all AnxA5 treatments by 49.4% (p=0.008), 53.3% (p=0.006) and 49.9% (p=0.011) respectively (fig 2D). The percentage medial macrophages was reduced by 61.2% (p=0.025) by 1.0 mg/kg AnxA5, the other dosages did not significantly affect monocyte/macrophage extravasation (fig 2E).

Together, these data indicate an important role for AnxA5 in low dosages in the prevention of leukocyte recruitment to injured arterial segments.

Figure 2

A

![Representative cross-sections of cuffed-femoral arteries of ApoE-/- mice treated with vehicle or 0.1, 0.3 or 1.0 mg/kg/d AnxA5 (leukocyte and macrophage staining, magnification 80x, arrows indicate positive staining)](image)

B

![Quantification of intimal adhering leukocytes (% of all cells)](image)

C

![Quantification of intimal macrophages (% of all cells)](image)

D

![Quantification of medial leukocytes (% of all cells)](image)

E

![Quantification of medial macrophages (% of all cells)](image)

Figure 2. Annexin A5 dose-dependently prevents leukocyte recruitment after vascular injury. Representative cross-sections of cuffed-femoral arteries of ApoE-/- mice treated with vehicle or 0.1, 0.3 or 1.0 mg/kg/d AnxA5 (leukocyte and macrophage staining, magnification 80x, arrows indicate positive staining) after 3d (A). Quantification of intimal adhering leukocytes (B) and macrophages (C) as percentage of all cells within the internal elastic lamina and medial infiltrated leukocytes (D) and macrophages (E) (%). Results indicated as mean±SEM, n=10. * p<0.05, ** p<0.01.
Annexin A5 dose-dependently prevents accelerated atherosclerosis development

The inflammation caused by cuff placement leads to an inflammation driven intimal hyperplasia. Therapeutic effectiveness of AnxA5 on (neo-)intima development was evaluated 14 days after cuff placement. Annexin A5 treatment did not affect plasma total cholesterol concentration (supplementary table I). Accelerated atherosclerotic lesion development was measured on sections stained with HPS and Weigert’s elastin (fig 3A). Vehicle-treated animals developed intimal thickening, resulting in luminal stenosis. Quantitative analysis displayed reduced intimal thickening (expressed as μm² per cross-section) after 0.1, 0.3 and 1.0 mg/kg AnxA5-treatment by 54.6% (p=0.041), 71.2% (p=0.012) and 66.9% (p=0.009) respectively (fig 3B). Intimal thickening was 38.1% more reduced (p=0.031) by 0.3 compared to 0.1 mg/kg AnxA5.

AnxA5 (0.3 and 1.0 mg/kg) also decreased the absolute medial surface area (μm²) by 30.1% (p=0.012) and 24.1% (p=0.025, fig 3C) and intima / media ratio by 62.3% (p=0.004) and 60.3% (p=0.007, fig 3D), although the lowest dose was ineffective. Furthermore, luminal stenosis (%) was reduced by 58.0% (p=0.001) and 58.8% (p=0.0004, fig 3E), identifying a potent role for AnxA5 in the control of inflammatory post-interventional vascular remodeling. Compared to 0.1 mg/kg, 0.3 mg/kg AnxA5 had increased protective effects on both the intima / media ratio (by 38.5%, p=0.016) and luminal stenosis percentage (by 33.2%, p=0.042). The total vessel wall diameter and luminal areas were both similar in all AnxA5 dosages, except for 1.0 mg/kg, which displayed 27.1% (p=0.043) reduced total vessel area (supplementary fig IA, B). IHC showed profound intravascular macrophages/foam cell areas, which co-localized with AnxA5 (supplementary fig IIA, B) staining at both 3d and 14d after surgery. AnxA5 in all dosages strongly reduced the accumulation of the percentage of macrophages/foam cell area (fig 4A) in the tunica media (fig 4B, p=0.0002, p=0.028 and p=0.0005 respectively) and in the tunica intima (fig 4C, p=0.002, p=0.011 and p=0.002 respectively) after 14d. The 78 kDa glucose regulated protein/BiP (GRP78) is an ER protein and associates permanently with mutant or defective incorrectly folded proteins, preventing their export from the ER lumen. ER stress including upregulation of GRP78 is present in unstable atherosclerotic lesions. We investigated if annexin A5 affected GRP78 expression in cuffed femoral arteries. AnxA5 in all dosages strongly reduced GRP78 BiP expressing cells in the media (fig 4D) by 50.2% (p=0.006), 66.3% (p=0.0006) and 68.0% (p=0.004) respectively, but not in the intima (fig 4E).
Figure 3

Annexin A5 reduces accelerated atherosclerosis development in a dose-dependent fashion. Representative cross-sections of cuffed arteries of ApoE−/− mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 (A) after 14d (HPS and Weigert's elastin staining, magnification 40x, arrows indicate internal elastic laminae). Quantification of intimal thickening (µm²) (B), medial area (µm²) (C), intima / media ratio (D) and luminal stenosis (%) (E). Results indicated as mean±SEM, n=10. * p<0.05, ** p<0.01.

Figure 4 next page.

Annexin A5 leads to a less-inflammatory phenotype with reduced intravascular signs of ER-stress. Representative cross-sections of cuffed arteries of ApoE−/− mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 (A) after 14d (macrophages and GRP78 BiP staining, magnification 40x, arrows indicate positive staining) and quantification of medial (B) and intimal (C) macrophage/foam cell area (%) and medial (D) and intimal (E) GRP78 BiP expression (%). Results indicated as mean±SEM, n=10. * p<0.05, ** p<0.01.


Discussion
This study demonstrates an important therapeutic role for AnxA5 in post-interventional intimal hyperplasia and accelerated atherosclerosis development. Association between AnxA5 SNPs and increased restenosis-risk in patients undergoing PCI was found. Systemic AnxA5 was effective in preventing intimal thickening and could dose-dependently reduce leukocyte and macrophage recruitment to injured arterial segments in ApoE\-/- mice in 0.3 and 0.1 mg/kg dosages. Finally, we demonstrate that sustained therapy reduces accelerated atherosclerosis with fewer infiltrated macrophages / foam cells and UPR-expressing cells in the injured arterial wall. Together, these date indicate high diagnostic and therapeutic potential for AnxA5 against post-PCI vascular remodeling.

Association between AnxA5 SNPs and restenosis development were investigated using a large study population that underwent PCI, the GENDER population. It has already been shown in this material that mutations in several genes associated with inflammation were associated to restenosis development (24). Our results demonstrate that SNPs rs4833229 and rs6830321 show significant association with increased risk for clinical restenosis (OR 1.29 and 1.35, fig 1A). This genetic variance in addition to plasma levels (19) would allow for
excellent stratification of patients that are most at risk for restenosis development, enabling individual tailor-made treatment strategy. Additionally, our results support the notion that genetic programming of not only pro-inflammatory mediators, but also the endogenous anti-inflammatory system exerts a significant role in post-interventional remodeling.

In this study, a perivascular cuff-mediated arterial injury model was applied, which allows for quick and reproducible lesion formation with continuous blood flow in a patent vessel segment, although the perivascular approach rather differs from clinical endovascular injury through balloon inflation and stent deployment during PCI. This perivascular approach could affect the amount of exposure of subendothelial thrombogenic material and thrombosis, which are important targets for AnxA5.

Therapeutic effects were shown to most likely result from local AnxA5 binding to activated cells in the injured vascular segment. Local AnxA5 can reduce adherence of platelets leukocytes and eventually prevent their inflammatory activation, with reduced signs of ER-stress and the UPR within these cells. We found reduced GRP78/BiP expression in the tunica media (fig 4D) but not in the intima (fig 4E). Prolonged intracellular cholesterol storage leads to increased ER stress in cells, which is more likely to occur in foam cells than in early monocyte/macrophages. In this study, such cells should predominantly be found among cells that have migrated towards the tunica media, which in turn may explain the difference between GRP78/BiP expression between the media and intima layers.

The fact that clearance of AnxA5 is much slower from the arterial wall than from plasma (30) and accumulates in the injured vascular wall after systemic injection (23), supports the hypothesis that AnxA5 could act anti-inflammatory in levels lower than originally investigated (<1.0 mg/kg). Current results confirm this, with AnxA5 already effective in reducing leukocyte (fig 2B) and macrophage (fig 2C) recruitment and intimal thickening (fig 3B) in dosages 3-10 times lower than previously investigated. This would favour clinical application, where undesired side-effects can be kept to a minimum.

In conclusion, this study shows that systemic AnxA5 treatment strongly influences post-interventional accelerated atherosclerosis development and can dose-dependently prevent vascular remodeling. AnxA5 has previously been successfully applied to diagnose atherosclerotic patients non-invasively (19). These results therefore may have important clinical implications. Immune-mediated interventions directed towards therapeutically controlling the leukocyte recruitment and vascular remodeling process could strongly benefit from systemic AnxA5, which could be applied in an early phase following revascularization or bypass grafting to prevent accelerated atherosclerosis development. AnxA5 SNPs could function as biomarkers in the assessment of restenosis risk in patients undergoing PCI, improving patient screening. Together, these data indicate high clinical potential for AnxA5 against post-interventional remodeling.

Disclosures
K. Pettersson is an employee of Athera Biotechnologies, Stockholm, Sweden. None of the other authors has any disclosure to report.

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Online supplements

Annexin A5 prevents post-interventional accelerated atherosclerosis development in a dose-dependent fashion

Materials and Methods

GENDER project

The GENetic DEterminants of Restenosis (GENDER) study was designed to investigate the association between genetic polymorphisms and clinical restenosis (1). In brief, it is a large multicenter prospective follow-up study conducted during 1999-2001 and comprised of patients treated successfully by percutaneous coronary intervention (PCI) for an acute coronary syndrome. Clinical restenosis was established during a nine-month follow-up period for death, myocardial infarction and target vessel revascularization (TVR), which occurred in 9.8% of all patients. Eight Single Nucleotide Polymorphisms (SNPs) included in the AnxA5 gene were extracted from the GENDER genome wide association study (GWAS) dataset (2) composed of 866 patients (295 cases that developed restenosis following PCI and 571 controls that did not develop restenosis after PCI). The GWAS was conducted using Illumina Human 610-Quad Beadchips (Illumina) and the infinium II assay, following the manufacturer’s instructions. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol, described in detail elsewhere (2). 

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software PLINK (3) was used to perform genetic association analysis. All p values were corrected for multiple testing. For linkage disequilibrium (LD) analyses in terms of $r^2$ and haplotype block delineation, we used Haploview software (4).

**Mice**

All experiments were approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC). ApoE$^{-/-}$ mice, purchased from the Jackson Laboratory (Bar Harbor) on a C57BL/6J background were used for these studies. All animals were 10-12 weeks at the start of a dietary run-in period before surgery. ApoE$^{-/-}$ mice were fed a Western-type diet containing 0.15% cholesterol (Lantmännen Lantbruk, diet R638). The diet was given three weeks prior to surgery and was continued throughout the entire experiment. All animals received food and water ad libitum during the experiment.

**Femoral artery cuff mouse model**

Mice were subjected to arterial femoral arterial cuff placement to induce intimal thickening and accelerated atherosclerosis development, as described previously (5-7). In brief, animals were anesthetized before surgery with a combination of intraperitoneally (IP)-injected Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen). The right femoral artery was isolated and sheathed with a rigid non-constrictive polyethylene cuff (Portex, 0.40mm inner diameter, 0.80mm outer diameter and an approximate length of 2.0mm). Animals received vehicle (0.9% sterile NaCl) or AnxA5 (Athera Biotechnologies AB) through IP injection. Three and 14 days after cuff placement, mice were anesthetized as before and euthanized. At sacrifice, blood was drawn in EDTA collection tubes (Sarstedt B.V.) and centrifuged at 6000 r.p.m. for 10 min at 4°C to obtain plasma, which was stored at -20°C. Next, the thorax was opened and mild pressure-perfusion (100mm Hg) with phosphate-buffered saline for 5 min by cardiac puncture in the left ventricle. After perfusion, the cuffed femoral artery was harvested, fixed in 3.7% formaldehyde in water (w/v) and paraffin-embedded. Serial cross-sections (5 μm thick) were made from the entire length of the artery for analysis.

**Biochemical analysis**

Total plasma cholesterol (Roche Diagnostics, kit 1489437) concentration was measured enzymatically before randomization at surgery.

**Quantification of cuffed femoral artery lesions**

Immunohistochemical (IHC) staining was performed using positive and negative tissue-specific controls as indicated by the antibody manufacturer. Samples were stained with hematoxylin-phloxine-saffron (HPS) and specific vessel wall composition was visualized for elastin (Weigert’s elastin staining) and with antibodies against GRP78 BiP (1:200, Abcam, to identify cells displaying signs of UPR), CD45 for leukocytes (1:200, Pharmingen), MAC3 for monocytes/macrophages/foam cells (1:200, BD Biosciences) and anti-annexin V for injected protein accumulation (1:100, BioVision). Using image analysis software (Leica Qwin), total cross-sectional medial area was measured between the external and internal elastic laminae; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina, as was GRP78 BiP+ surface area. The luminal stenosis is expressed as the percentage of surface area ($μm^2$) within the internal elastic lamina.
(comprised of the luminal and neointimal areas) that is taken up by neointimal tissue (in µm²). The number of leukocytes and monocytes/macrophages attached to the endothelium, within the neointimal tissue or infiltrated in the medial layer of the femoral arteries was quantified and is displayed as a percentage of the total number of present cells. All quantification in this study was performed on six equally spaced (150 µm distance) serial stained perpendicular cross-sections throughout the entire length of the vessel and was performed by blinded observers.

**Statistical analysis**

All data are presented as mean± standard error of the mean (SEM). Association between clinical outcome and individual SNPs was tested using an allelic association test. Groups were compared using a Mann-Whitney sum test for non-parametric data. Total plasma cholesterol concentrations in time were compared using a Wilcoxon matched pairs test. All statistical analyses were performed with SPSS 17.0 software for Windows or using Prism software. P-values <0.05 were regarded as statistically significant and are indicated with an asterisk (*).

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Annexin A5 prevents post-interventional accelerated atherosclerosis development in a dose-dependent fashion. Quantification of total vessel area (µm²) (A) and luminal area (µm²) (B) in ApoE-/- mice receiving vehicle or 0.1, 0.3 or 1.0 mg/kg AnxA5 after 14d. Results indicated as mean±SEM, n=10. * p<0.05, ** p<0.01, n.s. not significant.

Figure II. Co-localization of injected AnxA5 at macrophage (3d) and macrophage/foam cell (14d) areas after 3d (A) and 14d (B). Representative cross-sections of cuffed arteries of ApoE-/- mice receiving vehicle or 1.0 mg/kg AnxA5 (MAC3 and annexin A5 staining, magnification 80x, closed arrows indicate positive macrophage and AnxA5 staining, open arrows indicate projected macrophages in consecutive slides (5 µm distance) in annexin A5 stained femoral artery cross-sections to indicate co-localization).
Table I. Plasma cholesterol in mice undergoing annexin A5 dose-response investigations.

| Group                     | Total plasma cholesterol (mmol/L) |      |      |
|---------------------------|-----------------------------------|------|------|
|                           |                                   | Surgery | Sacrifice |
| Early time point (3d)     |                                   |      |      |
| Vehicle                   | 19.5±1.3                          | 25.0±0.8 |
| 1.0 mg/kg annexin A5      | 15.4±0.4                          | 19.8±1.6 |
| 0.3 mg/kg annexin A5      | 16.6±0.6                          | 20.0±1.7 |
| 0.1 mg/kg annexin A5      | 19.6±1.8                          | 22.8±2.2 |
| Late time point (14d)     |                                   |      |      |
| Vehicle                   | 23.7±2.7                          | 21.1±2.4 |
| 1.0 mg/kg annexin A5      | 18.0±2.7                          | 17.1±2.7 |
| 0.3 mg/kg annexin A5      | 19.9±4.1                          | 19.2±3.3 |
| 0.1 mg/kg annexin A5      | 19.3±1.6                          | 19.8±1.6 |

Plasma total cholesterol (mmol/L) of ApoE<sup>-/-</sup> mice receiving vehicle or annexin A5 (1.0, 0.3 or 0.1 mg/kg/d) trough IP injection, measured at surgery or at sacrifice (day 3 or 14). No significant differences were observed (mean±SEM, n=10).
“It always seems impossible until it's done”

Nelson Mandela