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Chapter 8

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-4Ig 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. T-cell subsets have been shown to play a vital role in this process. Unlike native atherosclerosis, their contribution to post-interventional remodeling and accelerated atherosclerosis development remains uninvestigated. Although local intimal hyperplasia consist predominantly of smooth muscle cells (SMCs) and connective tissue, 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.

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. 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. 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. 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.

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, leading to inflammation. Blocking of CD28-CD80/86 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 and production of tumor necrosis factor (TNF)-α, IL-2 and IFN-γ in vitro. Abatacept displays little immunogenicity, with <3% of patients developing an antibody response towards abatacept and is used to treat rheumatoid arthritis (RA) patients. Although the T-cell and CD28-CD80/CD86 co-stimulation 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. 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 ab-
tacept 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.

**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. 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.

**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. 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. 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. 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 IB), 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 IA,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.
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 athero
sclerosis 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%
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(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 in-
jured 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. 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.
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.

Abatacept affects systemic cytokine levels during accelerated atherosclerosis

Contribution of activated T-cell fractions to vascular remodeling severity is supported by the positive correlation (R²=0.61, p=0.002) between KLRG1+ CD4+ T-cells (%)
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.
and intimal thickening (µm²) (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±12.1 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±2.0 pg/ml in controls to 23.7±7.1 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 func-

![Figure 5. Profound CD4 T-cell contribution to post-interventional accelerated atherosclerosis development. (A) Positive correlation between KLRG1+ CD4 T-cell fractions (%) and intimal thickening (µm²) (n=6). Abatacept did not affect CD4 regulatory T-cell: effector T-cell ratio (B). Plasma IFNγ (pg/ml) was reduced after 14d following abatacept-treatment (C), whilst plasma IL-10 (pg/ml) was upregulated (D). Horizontal bars indicate median values, n=10. n.d. non-detectable. * p<0.05, ** p<0.01.](image-url)
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.

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 VIIIIE, 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.

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 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. 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. 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.\textsuperscript{22} showed that CD80\textsuperscript{+}/CD86\textsuperscript{+} LDL-receptor (r)\textsuperscript{−} mice developed decreased atherosclerotic lesions compared to LDL-r\textsuperscript{−} 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\textsuperscript{29} and this is essential for their suppressive function\textsuperscript{30,31}. Ait-Oufella et al.\textsuperscript{5} demonstrated the vital role of regulatory T-cells in native atherosclerosis development, using irradiated LDL-r\textsuperscript{−} mice receiving reconstituted bone-marrow from CD80\textsuperscript{−}/CD86\textsuperscript{−} 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\textsuperscript{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)\textsuperscript{23} and reduced concentration of IFN-γ (fig 5C) (baseline 9.8±4.6 pg/ml)\textsuperscript{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\textsuperscript{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.\textsuperscript{33} concerning the role of CD28\textsuperscript{−} activated T-cells and regulatory T-cells during acute coronary syndromes (ACS) in humans, as well as carotid stenosis\textsuperscript{34}, unstable angina\textsuperscript{35,36} and atherosclerosis development in both humans\textsuperscript{37} and animals in general\textsuperscript{38}. The function relevance of CTLA-4 expression for human peripheral T-cell has previously been shown by Hoff et al.\textsuperscript{39} and Yi-qun et al.\textsuperscript{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.\textsuperscript{41} and Körmendy et al.\textsuperscript{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.
Reference List

24. Coats AJ, Shewan LG. Statement on authorship and publishing ethics in the International Jour-


Online supplements

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) 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, 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. 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 (normocholesterolemic mice) after cuff placement, mice were anesthetized as before 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% 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 fluorochrome-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 (*).
Reference List


Table 1. Murine body weights at surgery and sacrifice. 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (gram)</th>
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<td></td>
<td>Surgery</td>
<td>Sacrifice</td>
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<tr>
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<tr>
<td>Abatacept</td>
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Figure 1. 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 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 (μm^2) and medial (C) and intimal (D) collagen area (μm^2) after 21 days. Horizontal bars indicate median values, n=10. ** p<0.01. 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).


T-cell co-stimulation in atherosclerosis

### Table 2

<table>
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<th>Group</th>
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<td>Surgery</td>
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<td>Anti-β gal IgG</td>
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<td>29.0±0.6</td>
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<tr>
<td>Anti-CTLA-4 IgG</td>
<td>30.4±0.7</td>
<td>30.1±0.8</td>
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</table>

Table 2. Murine body weights and plasma total cholesterol at surgery and sacrifice. Body weight (gram) and plasma total cholesterol (mmol/L) 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.

**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.

**Figure V.** Abatacept prevents systemic CD4 T-cell activation in ApoE3*Leiden mice. Quantification of splenic total cell count (*10^6 cells) (A) and CD4 T-cell fractions (%) (B) 14 days after surgery. Horizontal bars indicate median values, n=5.
Figure V. Abatacept prevents systemic CD4 T-cell activation in ApoE3*Leiden mice. Quantification of 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) and regulatory CD4+CD25+FoxP3+ T-cells (%) (D) in draining inguinal lymph nodes, 14 days after surgery. Horizontal bars indicate median values, n=5.
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T-cell co-stimulation in atherosclerosis

Figure VI. Abatacept does not affect draining inguinal lymph node T-cell activation status in hypercholesterolemic ApoE3*Leiden mice. Quantification of 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.