T-Cell Immunoglobulin and Mucin Domain 3 Acts as a Negative Regulator of Atherosclerosis

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Objective—Atherosclerosis is a chronic autoimmune-like disease in which lipids and fibrous elements accumulate in the arterial blood vessels. T cells are present within atherosclerotic plaques, and their activation is partially dependent on costimulatory signals, which can either provide positive or negative signals that promote T-cell activation or limit T-cell responses, respectively. T-cell immunoglobulin and mucin domain 3 (Tim-3) is a coinhibitory type 1 transmembrane protein that affects the function of several immune cells involved in atherosclerosis, such as monocytes, macrophages, effector T cells, and regulatory T cells. In the present study, we determined the role of Tim-3 in the development of atherosclerosis.

Approach and Results—Western-type diet–fed low-density lipoprotein receptor–deficient (LDLr−/−) mice were treated with an anti–Tim-3 antibody for 3 and 8 weeks. Anti–Tim-3 administration increased fatty streak formation with 66% and increased atherosclerotic plaque formation after 8 weeks with 35% in the aortic root and with 50% in the aortic arch. Furthermore, blockade of Tim-3 signaling increased percentages of circulating monocytes with 33% and lesional macrophages with 20%. In addition, anti–Tim-3 administration increased CD4+ T cells with 17%, enhanced their activation status, and reduced percentages of regulatory T cells with 18% and regulatory B cells with 37%.

Conclusions—It is known that Tim-3 acts as a negative regulator of both innate and adaptive immune responses, and in the present study, we show that anti–Tim-3 treatment augments lesion development, accompanied by an increase in the number of monocytes/macrophages and CD4+ T cells and by decreased regulatory T cells and regulatory B cells. (Arterioscler Thromb Vasc Biol. 2013;33:2558-2565.)

Key Words: atherosclerosis ■ immunology ■ inflammation ■ T cells ■ Tim-3

Costimulatory and coinhibitory molecules are important regulators of the immune system by fine-tuning innate and adaptive immune responses. Studies on atherosclerosis, a chronic inflammatory disease,1 show that modulation of costimulatory and coinhibitory pathways affects its development by regulating T-cell responses.2 Not only in secondary lymphoid tissues but also in the arterial wall, antigen-presenting cells, such as dendritic cells and macrophages, present antigens such as oxidized low-density lipoprotein (oxLDL) to T cells. The T cells can become either activated in the presence of costimulatory molecules or inhibited when T cells and antigen-presenting cells interact via coinhibitory molecules. Therefore, stimulating coinhibitory molecules may provide a novel therapeutic approach to prevent the activation or function of autoimmune immune cells in atherosclerosis. Previously, Gotsman et al3 showed that the coinhibitory pathway PD-1/PD-L1/2 inhibits proatherogenic T-cell responses and atherosclerosis because PD-L1/2−/− low-density lipoprotein receptor–deficient (LDLr−/−) mice develop significantly larger atherosclerotic lesions compared with LDLr−/− mice.4

T-cell immunoglobulin and mucin domain (Tim) proteins are type 1 transmembrane proteins expressed on various immune cells and are similar to PD-1/PD-L1/2 negative regulators of immune responses. Four functional TIM genes have been identified in the murine genome (TIM-1–4), whereas the human genome only contains 3 TIM genes (TIM-1, -3, and -4).4 The genes encoding Tim proteins are located on chromosome 11 (mouse) and chromosome 5 (human), which are associated with enhanced susceptibility to allergy and several autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) and diabetes mellitus.4

Tim-3 was first discovered as a specific marker for Th1 cells5 but is also expressed on a variety of immune cells such as natural killer (NK) cells, monocytes, macrophages, and mast cells.6 During innate immune responses, Tim-3 promotes inflammation via tumor necrosis factor-α (TNF-α)
secretion by monocytes and antigen-presenting cells and enhances macrophage clearance of intracellular pathogens. However, in adaptive immune responses, Tim-3 terminates interferon-γ-driven inflammation by inducing cell death of T cells after binding to its ligand galectin-9, a soluble molecule that is upregulated by interferon-γ. In addition, Tim-3 can induce regulatory T-cell (Treg) activity and induce macrophages. Blockade of the Tim-3–galectin-9 interaction might affect NK cell function during atherosclerosis. However, to date, the exact role of Tim-3 in atherosclerosis has not been investigated. In the present study, we therefore examined the role of Tim-3 during atherosclerosis development by treatment of LDLr−/− mice with a Tim-3 blocking antibody. This research provides novel information on the importance of the Tim-3 pathway in regulating immune responses and provides possibly new therapeutic targets to prevent atherosclerosis.

Materials and Methods

Results

TIM-3 Is Upregulated During Atherosclerosis Development

To determine TIM-3 expression in atherosclerotic lesions, LDLr−/− mice were fed a Western-type diet, and slightly constrictive perivascular collars were placed around the carotid arteries, which leads to the development of shear stress–induced atherosclerotic lesions at the proximal site of the collars. As shown in Figure 1A, TIM-3 expression was elevated after placement of collars around carotid arteries of LDLr−/− mice fed a Western-type diet (P<0.05), with the highest induction 10 weeks after collar placement (52-fold increase, P<0.05). To investigate which circulating cell types express Tim-3 and upregulate Tim-3 during Western-type diet feeding, we determined Tim-3 expression on the surface of several immune cells of mice fed a Chow or Western-type diet for 8 weeks with flow cytometry (n=5 per group; Figure 1B). Only minor populations of T cells, B cells, and neutrophils express Tim-3 on their surface, whereas larger populations of dendritic cells, monocytes, and NK cells express Tim-3. Western-type diet feeding significantly enhanced the percentage of Tim-3+ cells from 35.5±2.2% (Chow) to 45.2±2.5% in dendritic cells (P<0.05), from 28.8±2.3% to 46.3±3.3% in monocytes (P<0.01), and from 31.4±4.7% to 50.7±3.4% in NK cells (P<0.01). In addition, we determined whether Tim-3 is upregulated on the cell surface of these immune cells during atherosclerosis induction. As shown in Figure 1 in the online-only Data Supplement, hypercholesterolemia did not affect the amount of Tim-3 expressed on each cell.

Blocking Tim-3 Signaling Aggravates Atherosclerosis

Because interference of the Tim-3 signaling pathway aggravates autoimmune diseases such as EAE, type 1 diabetes...
mellitus, and graft-versus-host disease by dampening regulatory cells and enhancing monocyte and macrophage activation, we determined the role of Tim-3 in the initiation of atherosclerosis. We treated LDLr−/− mice twice a week with a Tim-3 blocking antibody (RMT3-23) and determined atherosclerotic lesion development after 3 and 8 weeks of Western-type diet feeding. Figure 2A and 2B shows representative cross-sections of lesions in the aortic valve area. Treatment with anti–Tim-3 significantly increased fatty streak formation in the aortic root with 66% (12.2±0.8×10^4 μm^2) compared with control mice (7.4±1.5×10^4 μm^2; P<0.05; Figure 2A). After 8 weeks of Western-type diet feeding, a 35% increase in the aortic root lesion size was observed in anti–Tim-3–treated mice (5.2±0.5×10^5 μm^2) compared with control mice (3.8±0.2×10^5 μm^2; P<0.05; Figure 2B). Previously, we showed that treatment with rat IgG, the isotype control for RMT3-23, did not alter atherosclerotic lesion size in comparison with PBS treatment. During the experiment, anti–Tim-3 treatment did not affect body weight and total plasma cholesterol levels (Figure IIA and IIB in the online-only Data Supplement). Furthermore, no differences were found in lesion extracellular matrix components (anti–Tim-3 mice: 11.1±1.5% and control mice: 12.1±1.8%; Figure 2C). To check whether anti–Tim-3 treatment also affects atherosclerosis development at another site of the vasculature, we determined lesion size in the aortic arch. As shown in Figure 3, anti–Tim-3 treatment (1.1±0.2×10^5 μm^2) aggravated atherosclerosis with 50% compared with control treatment (0.5±0.1×10^5 μm^2).

TIM-3 Blockade Enhances Circulating Monocytes and Lesional Macrophages

Monocytes play an important role in atherosclerosis development because monocytes migrate into the subendothelial space where they can differentiate into macrophages and eventually become foam cells. Previously, Tim-3 blockade has been associated with increased monocyte and macrophage activation and induces the expansion of macrophages. In our study, mice that received the Tim-3 antibody showed a 33% increase in circulating monocytes (control: 4.6±0.6% versus anti–Tim-3: 6.2±0.3%; P<0.01; Figure 4A). In mice, 2 subsets of monocytes with distinct patterns of surface markers and behaviors during inflammation have been described: CD11b^+Ly6G^-Ly6Chigh monocytes and CD11b^+Ly6G^-Ly6Clow monocytes. However, as shown in Figure IIIA and IIIB in the online-only Data Supplement, blockade of Tim-3 did not affect the frequency of these subsets. In line with increased circulating monocytes, lesional macrophage content was increased with 20% in anti–Tim-3–treated mice (49.0±2.3%) compared with control mice (40.7±2.9%; P<0.05; Figure 4B and 4C). An important key regulator of monocytes is monocyte chemotactic protein-1 (MCP-1), which enhances monocyte...
we did not observe differences in MCP-1 levels in serum of anti–Tim-3–treated and control-treated mice. However, oxLDL-stimulated macrophages exposed to anti–Tim-3 in vitro enhanced their secretion of MCP-1 in a dose-dependent manner (Figure 4E). Tim-3 can also induce a proinflammatory phenotype of macrophages independent from oxLDL because 50 ng/mL RMT3-23 significantly elevated MCP-1 secretion by macrophages (Figure IIIC in the online-only Data Supplement). In addition, we extracted RNA from aortic arches of control mice and anti–Tim-3–treated mice and determined MCP-1 expression with quantitative polymerase chain reaction. In line with our in vitro data, a trend toward an ≈2-fold increase of MCP-1 expression was observed in atherosclerotic lesions of anti–Tim-3–treated mice compared with control mice (Figure 4F).

Because TNF-α production is also known to be regulated by the Tim-3 pathway,7 we also determined the effect of Tim-3 blockade on TNF-α levels with ELISA. As shown in Figure IIID in the online-only Data Supplement, serum TNF-α levels did not differ between anti–Tim-3–treated and control mice, and although a trend toward increased TNF-α is observed in the supernatant of oxLDL-stimulated macrophages exposed to titrated amounts of the anti–Tim-3 antibody (Figure IIIE in the online-only Data Supplement), these differences were not significant.

Anti–Tim-3 Treatment Reduces Apoptosis of Lymphocytes

Tim-3 expressed on macrophages and dendritic cells is also involved in phagocytosis of apoptotic cells by recognizing apoptotic cells through the loop between the F and G beta strands in the IgV domain.19 In addition, Tim-3 can induce cell death of T cells. As shown in Figure 5A, we did not observe differences in lesion necrotic core content of anti–Tim-3 mice (24.5±2.7%) and control mice (28.7±2.5%). In addition, the percentage of apoptotic cells within the lesions did not significantly differ between anti–Tim-3 mice (0.70±0.21%) and control mice (0.80±0.18%; Figure 5B). Interestingly, a lower recruitment into the arterial wall.18 As shown in Figure 4D, we did not observe differences in MCP-1 levels in serum of anti–Tim-3–treated and control-treated mice. However, oxLDL-stimulated macrophages exposed to anti–Tim-3 in vitro enhanced their secretion of MCP-1 in a dose-dependent manner (Figure 4E). Tim-3 can also induce a proinflammatory phenotype of macrophages independent from oxLDL because 50 ng/mL RMT3-23 significantly elevated MCP-1 secretion by macrophages (Figure IIIC in the online-only Data Supplement). In addition, we extracted RNA from aortic arches of control mice and anti–Tim-3–treated mice and determined MCP-1 expression with quantitative polymerase chain reaction. In line with our in vitro data, a trend toward an ≈2-fold increase of MCP-1 expression was observed in atherosclerotic lesions of anti–Tim-3–treated mice compared with control mice (Figure 4F).

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Figure 5. Necrotic areas within atherosclerotic lesions of anti–T-cell immunoglobulin and mucin domain 3 (Tim-3) and control mice were determined (A). The necrotic core was defined as the acellular, debris-rich plaque area as percentage of total plaque area. Apoptosis was defined by TUNEL-positive nuclei as percentage of total number of cells present in the atherosclerotic lesions (B). The percentage of apoptotic lymphocytes in the spleen was determined with an Annexin-V/PI staining and analyzed with flow cytometry (C). TUNEL indicates terminal deoxyribonucleotidyl transferase dUTP nick end labeling; and PI, propidium iodide. **P<0.01.
percentage of splenic lymphocytes isolated from anti–Tim-3 mice (26.2±0.9%) underwent apoptosis than did control spleenocytes (32.7±1.3%; \(P<0.01\); Figure 5C).

**Reduced Tregs and Regulatory B Cells in Anti–Tim-3–Treated Mice**

Several studies showed that interactions of Tim-3 with galec-tin-9 are essential for the generation of Tregs. Tregs play an important role in the regulation of T-cell–mediated immune responses through suppression of T-cell proliferation and cytokine production. Therefore, increased Treg numbers may be beneficial for patients with atherosclerosis. In accordance with literature, we observed an 18% reduction of circulating Tregs within the CD4+ T-cell population in anti–Tim-3–treated mice (8.8±0.2%) compared with control mice (10.8±0.6%; \(P<0.05\); Figure 6A) and a 17% reduction of Tregs in the spleen of anti–Tim-3–treated mice (5.8±0.3%) compared with control mice (7.0±0.4%; \(P<0.05\); Figure 6A). We also extracted RNA from atherosclerotic lesions in the aortic arches of control mice and anti–Tim-3–treated mice. As shown in Figure 6B, anti–Tim-3–treated mice showed a 70% reduction (\(P=0.001\)) in Foxp3 mRNA expression compared with control mice.

Another regulatory cell type is the regulatory B cell (Breg), which is an interleukin (IL)-10 producing B cell that induces T-cell–mediated immune responses through suppression of T-cell proliferation and cytokine production. Therefore, increased Treg numbers may be beneficial for patients with atherosclerosis. In accordance with literature, we observed an 18% reduction of circulating Tregs within the CD4+ T-cell population in anti–Tim-3–treated mice (8.8±0.2%) compared with control mice (10.8±0.6%; \(P<0.05\); Figure 6A) and a 17% reduction of Tregs in the spleen of anti–Tim-3–treated mice (5.8±0.3%) compared with control mice (7.0±0.4%; \(P<0.05\); Figure 6A). We also extracted RNA from atherosclerotic lesions in the aortic arches of control mice and anti–Tim-3–treated mice. As shown in Figure 6B, anti–Tim-3–treated mice showed a 70% reduction (\(P=0.001\)) in Foxp3 mRNA expression compared with control mice.

To determine whether anti–Tim-3 treatment affects the suppressive function of Tregs and Bregs, we isolated Tregs and Bregs and added titrated amounts to CD4+ T cells. Their suppressive capacity was determined with a Ki-67 staining or tritium thymidine incorporation, respectively. We also isolated RNA from the Tregs and analyzed relative mRNA expression of important molecules that are associated with Treg-mediated T-cell suppression: CTLA-4 and GITR. As shown in Figure IV in the online-only Data Supplement, interference in Tim-3 signaling does not affect the inhibitory function of Tregs and Bregs.

In addition, a 55% decrease in all IL-10–producing cells was measured in spleens of anti–Tim-3–treated mice (3.5±1.0%) compared with the control group (7.9±1.5%; \(P<0.05\); Figure 6D).

**Tim-3 Blockade Increased the Percentage and Activation Status of CD4+ T Cells**

Because Tregs control CD4+ T cells and Tim-3 blockade reduces Tregs, we determined the percentage of CD4+ T cells. As expected, we found significantly increased CD4+ T cells in the spleen of anti–Tim-3–treated mice (25.1±1.4%) compared with control mice (21.4±0.6%; \(P=0.05\); Figure 6E). Furthermore, blockade of the Tim-3 pathway significantly enhanced the activation status of CD4+ T cells. CD4+CD62Llow effector T cells were increased with 35% in anti–Tim-3–treated mice (12.3±1.2% versus control: 9.1±0.4%; \(P<0.05\)), and memory T cells were increased with 27% in anti–Tim-3–treated mice (6.8±0.5%) compared with control mice (5.4±0.1%; \(P<0.05\); Figure 6F). Because Tim-3 is particularly associated with the regulation of Th1 and Th17 responses, we also determined the percentage of Th1 (CD4+T- bet+), Th2 (CD4+GATA-3+), and Th17 (CD4+ROR\(\gamma\)t+) cells in the spleen and their expression in atherosclerotic lesions of the aortic arch. Splenic and lesional Th1, Th2, and Th17 cells (Figure VA in the online-only Data Supplement and Figure 6B, respectively) were not affected by the anti–Tim-3 treatment. However, CD4+ T cells in the spleen from anti–Tim-3–treated mice produced more IL-17 (4.6±0.7% versus control: 3.0±0.2%; \(P<0.05\); Figure VB in the online-only Data Supplement). Notably, there were no changes in the absolute numbers of total spleen cells (anti–Tim-3: 78.4±11.0×10⁶ cells versus control: 76.6±7.0×10⁶ cells; \(P=0.72\)).

To evaluate the net effect of anti–Tim-3 treatment on soluble mediators of inflammation, we also performed a multiplex bead assay on supernatant of αCD3/CD28 stimulated splenocytes and on serum samples of anti–Tim-3–treated mice and control mice to the cytokine environment. As shown in Figure 6, after being euthanized, blood and spleen cells were isolated and stained for CD4+CD25+Foxp3+ regulatory T cells (Tregs; A) and analyzed by flow cytometry (n=5 per group). Relative mRNA expression of transcription factors representative for T-cell subsets is measured in atherosclerotic lesions of the aortic arch (n=5 per group; B). Spleen and peritoneal cells were stained for regulatory B cells (Bregs), which were quantified as CD5+CD1d+CD21+ cells and CD5+CD1d+ cells, respectively (C). The percentage of all interleukin (IL)-10–producing cells in the spleen was determined by flow cytometry (D). CD4+ T cells were determined in the spleen (E). Effector (CD4+CD62Llow) and effector memory cells (CD4+CD44hiCD62Llow) were also determined in the spleen (F).

*\(P<0.05\); **\(P<0.001\).
VC and VD in the online-only Data Supplement, no large variances between the cytokine profiles of control (white bars) and anti–Tim-3 mice (black bars) were observed. The cytokines IL-13 and IL-17 were elevated in anti–Tim-3–treated mice (P<0.05). Anti–Tim-3–treated mice showed a large increase in serum IL-6, a proinflammatory/proatherogenic cytokine.

**Anti–Tim-3 Induces a Proatherogenic B-Cell Profile**

To assess the effect of Tim-3 blockade on humoral responses, we analyzed B1 and B2 cell subsets in the circulation, spleen, and peritoneum. As shown in Figure VI in the online-only Data Supplement of the revised article, anti–Tim-3 treatment reduced the percentage of B1 cells (CD4+CD5+IgM+ cells) by 78% in the peritoneum (0.7±0.2 versus control: 3.0±0.2%; P<0.05), whereas B2 cells (IgM+IgD+ within CD19+ cells) were increased with 61% (50.1±6.4% versus control: 31.1±1.6%; P<0.001). No differences in B-cell subsets were observed in blood and spleen. In addition, we determined oxLDL-specific antibodies in serum of anti–Tim-3–treated mice and control mice. Anti–Tim–3–treated mice showed elevated levels of IgM, IgG1, and IgG2b compared with control mice, which suggests that anti–Tim-3 treatment promotes the overall activation status of B cells.

**Discussion**

Acute cardiovascular syndromes are a major cause of death in Western society and are generally triggered by rupture of an atherosclerotic plaque. In atherosclerosis, an imbalance between pro- and anti-inflammatory T cells exists, with increased numbers of the first. Restoration of this balance by modulation of costimulatory and coinhibitory molecules may provide a very promising strategy to prevent cardiovascular disease. The role of coinhibitory Tim-3 has been established in several autoimmune diseases, such as EAE, graft-versus-host disease, and type 1 diabetes mellitus; however, the contribution of Tim-3 to atherosclerosis development remains to be elucidated.

In the present study, we show that TIM-3 is expressed within the atherosclerotic lesion and increases during Western-type diet feeding. Furthermore, we found that Tim-3 was expressed on large populations of monocytes, dendritic cells, and NK cells, whereas only a few T cells, B cells, and neutrophils expressed Tim-3 on their surface. Previously, Hou et al showed that patients with atherosclerosis have increased Tim-3+ NK cells compared with healthy controls. In line with these findings, we observed that Western-type diet feeding increased not only the percentage of Tim-3+ NK cells, but also the percentage of Tim-3+ monocytes and Tim-3+ dendritic cells, cell types that largely contribute to the inflammatory process of atherosclerosis.

To investigate the contribution of Tim-3 to atherosclerosis development, we treated Western-type diet–fed LDLr−/− mice with an anti–Tim-3 antibody for 3 and 8 weeks. After 3 weeks of Western-type diet feeding, we observed already a significant 66% increase in the aortic root lesion size in anti–Tim-3–treated mice compared with control mice. Blockade of Tim-3 for 8 weeks increased atherosclerosis development with 35% in the aortic root and with 50% in the aortic arch compared with control treatment, independent of plasma cholesterol levels. These data illustrate that anti–Tim-3 treatment increased lesion development already from an early stage. Whereas there was no difference in lesion stability, lesions of anti–Tim–3–treated mice contained significantly more macrophages than lesions of control mice. In agreement, circulating monocytes were enhanced in anti–Tim-3 mice, and because monocytes migrate into the arterial wall and differentiate into macrophages that can take up oxLDL, this may result in enhanced lesional macrophages. To mimic foam cell responses in the lesion, we exposed oxLDL-loaded macrophages to anti–Tim-3 in vitro and showed that blockade of Tim-3 enhanced MCP-1 secretion, which may be responsible for increased monocyte infiltration and subsequent elevated lesional macrophages in anti–Tim–3–treated mice. In line with our in vitro data, treatment with anti–Tim-3 resulted in an ≈2-fold increase of MCP-1 expression in atherosclerotic lesions as compared with control mice, suggesting that MCP-1 may indeed be, at least partly, responsible for the increase in macrophage content. These data are in accordance with studies that show an essential role for Tim-3 in monocyte and macrophage function. Money et al appointed increased macrophage numbers and activation as the major cause of exacerbated EAE in mice treated with a blocking anti–Tim-3 antibody. Frisancho-Kiss et al showed that anti–Tim-3 treatment during the innate response to viral infection in BALB/c mice increases macrophages and their activation in the heart, resulting in increased inflammatory heart disease, and Zhang et al found that Tim-3 regulated pro- and anti-inflammatory cytokine expression in human CD14+ monocytes.

Because Tim-3 signaling has previously been associated with apoptosis, we determined the percentage of apoptotic cells and necrotic core in lesions of anti–Tim–3 mice. In early lesions, apoptosis of macrophages limits lesion cellularity and suppresses lesion progression. However, in advanced stages, it induces necrotic core formation, which makes lesions susceptible for rupture. Therefore, it has to be taken into account that Tim-3 signaling could be beneficial in initial stages of atherosclerosis, but in later stages of atherosclerosis could promote plaque rupture. In our study, we do not observe differences in apoptosis or necrotic core content in the lesions of anti–Tim–3–treated mice and Tim-3 signaling also enhances Tregs, which have been shown to promote lesion stability in advanced stages of atherosclerosis. More specifically, it has been reported that Tim-3–galectin-9 interaction promotes apoptosis of T cells. We do see reduced apoptosis of lymphocytes in anti–Tim–3–treated mice, which in turn could be responsible for the increase in CD4+ T cells.

Moreover, Tim-3 has also been implicated in regulating proinflammatory T-cell responses by promoting Treg function. Tregs dampen the immune response in atherosclerosis by secreting anti-inflammatory cytokines, such as IL-10, and by directly inhibiting effector T-cell proliferation. Seki et al reported that Tim-3 activation by galectin-9 resulted in an induction of Tregs, and Frisancho-Kiss et al showed that anti–Tim-3 administration reduces Treg populations in the heart during acute myocarditis. In addition, blockade of the Tim-3 pathway accelerated type 1 diabetes mellitus in non-obese diabetic mice in part by dampening Treg function.13
In accordance with these findings, we also observed reduced Treg levels in spleen, blood, and atherosclerotic lesions after anti–Tim-3 administration, which contributed to the exacerbation of atherosclerosis. In addition, Bregs were significantly decreased with 37% in the spleen and with 44% in the peritoneum of anti–Tim-3–treated mice. The exact role of Bregs in atherosclerosis has not been clarified yet; however, Bregs secrete IL-10 and thereby inhibit secretion of proinflammatory cytokines and support Treg differentiation, which indicates a protective role for Bregs in atherosclerosis. The atheroprotective role of IL-10 has already been shown by Von Der Thüsen et al23 who showed that IL-10 overexpression reduced atherosclerosis in LDLr−/− mice. Furthermore, adoptive transfer of Bregs inhibited the initiation of EAE in B16 mice immunized with MOG peptides,24 and Mauri et al25 demonstrated that a transfer of IL-10–producing B cells into DBA mice prevented rheumatoid arthritis development and ameliorated established arthritis. Finally, the percentage of IL-10–producing splenocytes was decreased with 55% after anti–Tim-3 treatment, which is in line with decreased Tregs and Bregs in anti–Tim-3 mice, which exert their function in part via IL-10 secretion. Notably, interference in Tim-3 signaling solely affects Tregs and Bregs by reducing their numbers because anti–Tim-3 treatment did not affect their inhibitory function.

Reduced Tregs promoted the percentage of total and activated peripheral CD4+ T cells in anti–Tim-3–treated mice. Whereas Th1, Th2, and Th17 cells were unaffected in the spleen and atherosclerotic lesions of anti–Tim-3–treated mice, CD4+ T cells of anti–Tim-3–treated mice produced more IL-17. This finding is in line with previous studies that showed that abrogation of the Tim-3 pathway induces IL-17 production.26,27 The role of IL-17 in atherosclerosis is controversial but mainly considered proatherogenic because the expression of IL-17 and RORγt is correlated to plaque size and exogenous IL-17 promotes the formation of atherosclerotic lesions in apolipoprotein E–deficient mice.28 To study whether Tim-3 affects atherosclerosis through modulation of humoral immune responses, we determined B-cell subsets and oxLDL–specific antibody responses. Anti–Tim-3 treatment reduced the percentage of peripheral B1 cells by 78%, whereas B2 cells were increased by 61%. B1 cells are considered antiatherogenic,29 whereas B2 cells promote atherosclerotic lesion development,30,31 illustrating that anti–Tim-3 induces a proatherogenic B-cell profile. In addition, elevated levels of oxLDL–specific antibodies were found in serum of anti–Tim-3–treated mice, which suggests that anti–Tim-3 treatment promotes the overall activation status of B cells.

In the present study, we are the first to provide direct evidence that the Tim-3 pathway is involved in the development of atherosclerosis. We show that Tim-3 acts as a negative regulator of atherosclerosis because blockade of Tim-3 augmented atherosclerotic lesion development, which was accompanied by increased circulating monocytes and leisolal macrophages and by decreased Tregs and Bregs, which subsequently induced the activation of CD4+ T cells. In the future, the use of lymphocyte-deficient mice would be a very interesting approach to study the role of the adaptive immune system in anti–Tim-3–mediated aggravation of atherosclerosis. Moreover, approaches to promote the Tim-3 pathway, such as treatment with agonistic Tim-3 antibodies or galectin-9, may represent novel therapeutic strategies to inhibit atherosclerotic lesion development and prevent cardiovascular diseases.

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Disclosures
None.

References
Acute cardiovascular syndromes are a major cause of death in Western society and are generally triggered by rupture of an atherosclerotic plaque. Besides lipid accumulation, an imbalance between pro- and anti-inflammatory responses plays a key role in atherosclerotic plaque development. Therefore, restoration of this balance by inhibition of proinflammatory responses and by inducing suppressor cells has great therapeutic potential to prevent cardiovascular disease. In this study, we are the first to provide direct evidence that T-cell immunoglobulin and mucin domain 3, a coinhibitory molecule that can terminate T-cell survival, cell cycle progression, and differentiation of naive T cells to effector and memory T cells, is involved in the development of atherosclerosis. Western-type diet–fed low-density lipoprotein receptor–deficient mice treated with an anti–T-cell immunoglobulin and mucin domain 3 antibody showed aggravated atherosclerosis accompanied by enhanced monocytes/macrophages and effector T cells and reduced regulatory T and B cells. This study shows that promoting the T-cell immunoglobulin and mucin domain 3 pathway may represent a novel therapeutic strategy to inhibit atherosclerotic lesion development and prevent cardiovascular diseases.
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SUPPLEMENTAL MATERIAL
Supplemental Figure I. Protein levels (MFI) of Tim-3 were determined on immune cells in the circulation of mice fed a Chow diet (n=5) or a Western-type diet (n=5) for 8 weeks with flow cytometry.

Supplemental Figure II. No differences in weight and total cholesterol levels after anti-Tim-3 treatment during atherosclerosis development. LDLr<sup>−/−</sup> mice received Western-type diet for 8 weeks and were treated i.p. with anti-Tim-3 (black circles, n=12) or PBS (open circles, n=11). During the experiment, mice were weighed (A) and blood was taken by tail vein bleeding. Total cholesterol concentration was determined within the serum (B).
**Supplemental Figure III.** Atherosclerosis was induced in LDLr^{−/−} mice by feeding a Western-type diet for 8 weeks. Mice were treated with anti-Tim-3 (n=12) or sterile PBS (n=11) twice a week. At sacrifice, blood cells were isolated (n=5 per group) and stained for CD11b^{+}Ly6G^{−}Ly6C^{high} monocytes (A) and CD11b^{+}Ly6G^{−}Ly6C^{low} monocytes (B). BM-derived macrophages were stimulated for 24 hours with various concentrations of anti-Tim-3. MCP-1 levels in the supernatant were determined with ELISA (C). TNFα concentrations were measured in the serum of control (n=11) and anti-Tim-3-treated mice (n=12) with ELISA (D). BM-derived macrophages were stimulated for 24 hours with 2.5 µg/ml oxLDL in the presence of various concentrations of anti-Tim-3. TNFα levels in the supernatant were determined with ELISA (E). *** P<0.001

**Supplemental Figure IV.** Atherosclerosis was induced in LDLr^{−/−} mice by feeding a Western-type diet for 8 weeks. Mice were treated with anti-Tim-3 (n=12, black bars) or sterile PBS (n=11, white bars) twice a week. At sacrifice, Tregs were isolated and titrated amounts were added to CD4^{+} T cells. The cells were cultured for 72 hours in the presence of αCD3/CD28 stimulation and stained for CD4 and Ki-67, a proliferation marker. Positive cells were assessed with flow cytometry (A). Relative mRNA levels of CTLA-4 and GITR were determined in isolated Tregs with RT-PCR (B). A suppression assay was performed to determine the suppressive capacity of the expanded Bregs by measuring the proliferation of CD4^{+} T cells (C). Data are shown as the mean counts per minute (cpm) of quintuplicate cultures.
**Supplemental Figure V.** Atherosclerosis was induced in LDLr<sup>-/-</sup> mice by feeding a Western-type diet for 8 weeks. Mice were treated with anti-Tim-3 (n=12, black bars) or sterile PBS (n=11, white bars) twice a week. At sacrifice, splenocytes were isolated, stained for CD4, T-bet (Th1), GATA-3 (Th2) and RORγt (Th17) and analyzed with flow cytometry (A). IFN-γ and IL-17 secretion by these CD4<sup>+</sup> T cells was determined by flow cytometry (B). A multiplex bead assay was performed on supernatant of αCD3/CD28 stimulated splenocytes (C) and on serum samples (D) of anti-Tim-3-treated mice and control mice (n=5 per group). * P<0.05, ** P<0.01

![Graphs showing CD4+ T cell analysis](image)

**Supplemental Figure VI.** Atherosclerosis was induced in LDLr<sup>-/-</sup> mice by feeding a Western-type diet for 8 weeks. Mice were treated with anti-Tim-3 (n=12, black bars) or sterile PBS (n=11, white bars) twice a week. At sacrifice, blood, spleen and peritoneal cells were isolated (n=5 per group) and stained for B1 cells (CD4<sup>-</sup>CD5<sup>+</sup>IgM<sup>-</sup>, A) and B2 cells (IgM<sup>lo</sup>IgD<sup>hi</sup> within CD19<sup>+</sup> cells, B) and analyzed with flow cytometry. OxLDL-specific IgM, IgG1, IgG2a and IgG2b production was detected in serum of control and anti-Tim-3-treated mice (n=5 per group) (C).

* P<0.05, ** P<0.01, *** P>0.001

![Graphs showing B cell analysis](image)
Material and Methods

Animals
Female LDLr deficient (LDLr−/−) mice, 10-12 weeks old, were obtained from Jackson Laboratories. The animals were kept under standard laboratory conditions and were fed either a chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Diet and water were provided ad libitum. All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines.

Atherosclerosis
Atherosclerosis was induced in LDLr−/− mice by feeding a Western-type diet for 3 or 8 weeks. Mice were treated twice a week i.p. with 250 µg anti-Tim-3 antibody (RMT3-23, n=6 and n=12) or sterile PBS (n=5 and n=11) for 3 or 8 weeks, respectively. At week 3 and 8 mice were sacrificed and tissues were harvested after in situ perfusion using PBS. Tissues for histology were fixated in Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Tissues for RNA analysis were frozen in nitrogen and stored at -20 °C until further use.

TIM-3 expression during atherosclerosis
After 2 weeks of Western-type diet, atherosclerosis was induced as previously described by collar placement (2 mm long, diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding. Mice were sacrificed at 0, 2, 4, 6, 8 and 10 weeks after collar placement and tissues were harvested after in situ perfusion using PBS and subsequent perfusion using Zinc Formal-Fixx (Shandon Inc. Pittsburg, USA). Carotid arteries (n = 4-6 per timepoint) were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SYBR green technology. The following primer pair was used for Tim-3: 5’-TGGAGTGAGGTCTCTGTGGGT-3’ and 5’-GCTCCTGCATTGGCAACCCTCC-3’. The following primers were used as endogenous references: 5’-GGACCCGAGAAGACCTCCTT-3’ and 5’-GCACATACTCAGAATTTCAATG-3’ for acidic ribosomal phosphoprotein PO (36B4), 5’-TTGCTCGAGATGTCATGAAGGA-3’ and 5’-AGCAGGTCAGCAAGAGACTTATAG-3’ for hypoxanthine phosphoribosyltransferase (HPRT), 5’-AGCAGGTCAGCAAGAGACTTATAG-3’ and 5’-AGCAGGTCAGCAAGAGACTTATAG-3’ for Rps13 and 5’-AACCCTGAAAGATGAGCAGCAGAT-3’ and 5’-CACAGGCTGGATGGCTCGTA-3’ for α-actin.

Serum cholesterol levels
During the experiments, mice were weighed and blood samples were obtained by tail vein bleeding. The total cholesterol levels in serum were determined at week 0, 2, 4, 6 and 8 after start of the Western-type diet feeding. The concentrations of serum cholesterol were determined using enzymatic colorimetric procedures (Roche/Hitachi, Mannheim, Germany). Precipath (Roche/Hitachi) was used as an internal standard.

Histological analysis and morphometry
Cryosections of the aortic root (10 µm) were made and stained with Oil-red-O to determine lesion size. Lesion extracellular matrix components were determined with a Masson’s Trichrome staining. Furthermore, corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (Moma-2, monoclonal rat IgG2b, diluted 1:1000). Goat anti-rat IgG alkaline phosphatase conjugate (dilution 1:100) was used as a secondary antibody and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as enzyme substrates. The necrotic core was defined as the a-cellular, debris-rich plaque area as percentage of total plaque area. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of lesions was performed to determine the rate of apoptosis in the atherosclerotic lesions using the In Situ Cell


Death Detection Kit (Roche Diagnostics, Mannheim, Germany). TUNEL-positive nuclei were visualized with Nova Red (Vector Laboratories), and sections were counterstained with 0.3% methylgreen. TUNEL positive nuclei were counted manually and the percentage of apoptotic cells was determined as percentage of total cells. In addition, the aortic arch and its main branch points were excised, fixed, and embedded in paraffin. Longitudinal sections of the aortic arch (4 µm) were analyzed for lesion extent with a hematoxylin and eosin staining. Morphology was studied using a Leica DM-RE microscope and LeicaQwin software (Leica imaging systems, Cambridge, UK).

**Flow cytometry**

At sacrifice, blood, spleen and peritoneal cells were isolated (n=5 per group). Single cell suspensions were obtained by squeezing the organs through a 70 µm cell strainer. Red blood cells were removed from blood and splenocytes using erythrocyte lysis buffer (0.15 M NH₄Cl, 10 mM NaHCO₃, 0.1 mM EDTA, pH 7.3). Different immune cells were analyzed for their Tim-3 expression or for their frequency with flow cytometry: T cells (CD4, CD8), B cells (CD19), DCs (CD11c), neutrophils (CD11b'Ly6G'), monocytes (CD11b'Ly6G', CD11b'Ly6G'Ly6C<sup>high</sup>, CD11b Ly6G'Ly6C<sup>low</sup>), NK cells (CD3'NK1.1'), B cells (B1 cells: CD4'CD5'IgM<sup>+</sup>, B2 cells: CD19'IgM<sup>+</sup>IgD<sup>+</sup>, Bregs; splenic subset: CD5'CD1d<sup>+</sup>CD21', peritoneal subset: CD5'CD1d<sup>-</sup>), Tregs (CD4'CD25<sup>+</sup>Foxp3<sup>+</sup>), Th1 cells (CD4'T-bet<sup>+</sup>, CD4'IFN-γ<sup>+</sup>), Th2 cells (CD4'GATA-3<sup>+</sup>), Th17 cells (CD4'ROγt<sup>+</sup>, CD4'IL-17<sup>+</sup>) and IL-10<sup>+</sup> cells. To detect T cell subsets and cytokine producing cells, cells were fixed and permeabilized according to manufacturer’s protocol (eBioscience, Vienna). Subsequently, the cells were stained for transcription factors or cytokines. All antibodies were purchased from eBioscience (Vienna) and Beckton Dickinson (Mountain View, CA). To detect apoptotic cells, an Annexin-V/PI staining was performed on splenocytes according to manufacturer’s protocol (eBioscience, Vienna). FACS analysis was performed on a FACSCantoII (Beckton Dickinson). Data were analyzed using FACSDiva software (Beckton Dickinson).

**MCP-1 and TNFα determination in serum and supernatant of oxLDL-loaded macrophages**

To detect MCP-1 and TNFα in serum an ELISA was performed according to manufacturer’s protocol (MCP-1; eBioscience, Vienna, TNFα; Beckton Dickinson) and absorbance was detected at 450 nm. To determine MCP-1 and TNFα secretion by oxLDL-loaded macrophages, bone marrow cells were harvested from the femur and tibia of C57BL/6J mice and were cultured for 7 days in complete RPMI supplemented with M-CSF (L929 supernatant) to obtain macrophages. Immature macrophages were stimulated with or without copper-oxidized LDL (2.5 µg/ml) in the absence or presence of 0, 2, 10, 25 or 50 µg/ml anti-Tim-3 (RMT3-23) for 24 hours. MCP-1 and TNFα production was measured in the supernatant with ELISA as described above.

**Suppression assays Tregs and Bregs**

To determine the inhibitory capacity of Tregs and Bregs, Tres and Bregs were isolated from splenocytes of Western-type diet fed LDLr<sup>−/−</sup> mice treated with PBS (n=3) or anti-Tim-3 (n=3) with magnetic beads (Miltenyi Biotec) according to manufacturer’s protocol. 5×10<sup>5</sup> CD4<sup>+</sup> T cells isolated with a CD4<sup>+</sup> T cell enrichment kit (BD Biosciences) were plated out per well of a 96-well plate with or without titrated amounts of isolated Tregs and Bregs. Cells were activated with anti-CD3 and anti-CD28 (1 µg/ml) for 72 hours. CD4<sup>+</sup> T cells from the coculture with Tregs were stained for CD4 and Ki-67, a proliferation marker, and proliferation was determined by flow cytometry. CD4<sup>+</sup> T cells from the coculture with Bregs were pulsed with 3<sup>H</sup>-thymidine (0.5 µCi/well) on day 3. Proliferation was assessed 16 hours later using a liquid scintillation counter. All results are expressed as the mean counts per minute (cpm) of quintuplicate cultures. In addition, RNA was isolated from the isolated Tregs using the guanidium isothiocyanate (GTC) method and reverse transcription (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis of CTLA-4 and GITR (Table I) was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SYBR green technology.
Table I. Sequences of primers for RT-PCR

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CTLA-4</td>
<td>GAACCTGTGTTACCACCGCCAT</td>
<td>CCCAAGCTTACGCGACAGGA</td>
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<tr>
<td>GITR</td>
<td>GCCCTAGTGCTGCCAGTCACTC</td>
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T cell subsets and MCP-1 expression in atherosclerotic lesions
To determine T cell subsets present in the adventitia/plaque, RNA was extracted from the aortic arches of control mice (n=5) and anti-Tim-3-treated mice (n=5) using the guanidium isothiocyanate (GTC) method and reverse transcription (RevertAid M-MuLV reverse transcriptase) as described above. The expression of T-bet (Th1 cells), GATA-3 (Th2 cells), RORγt (Th17 cells), Foxp3 (Tregs) and MCP-1 were determined (Table II).

Table II. Sequences of primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>AGTGACTGCTACCAGAACGAGAG</td>
<td>CCAGGTGGCCAGGGGACACT</td>
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<td>GATA-3</td>
<td>CGGGTCGCGATGAAAGTCCGAGG</td>
<td>ATTTTATGGTAGAGTCCGCAGCAT</td>
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<td>RORγt</td>
<td>GCCGCTTACGGCTCTCATGG</td>
<td>TCCACACCACGATGGGATCCT</td>
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<td>Foxp3</td>
<td>GGAGCCGAGCTAAAAGC</td>
<td>TGCCTTCGTCGAGCAGCAG</td>
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<tr>
<td>MCP-1</td>
<td>CTGAAGCCAGCTCCTCCTCCTCCTC</td>
<td>GGTGAATGAGTAGCAGGATGC</td>
</tr>
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</table>

Multiplex cytokine assay
To evaluate the net effect of anti-Tim-3 treatment on soluble mediators of inflammation, a multiplex cytokine assay (Invitrogen) was performed on supernatant of anti-CD3/CD28 stimulated splenocytes and serum of anti-Tim-3-treated mice and control mice fed a Western-type diet for 8 weeks. The following cytokines were screened according to manufacturer’s protocol: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17 and KC. Standard curves for each cytokine were generated by using the reference cytokine concentrations supplied in this kit.

Statistical analysis
All data are expressed as mean±SEM. An unpaired two-tailed student T-test was used to compare normally distributed data between two groups of animals. Probability values of P<0.05 were considered significant.