Blockade of Tim-1 and Tim-4 Enhances Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice


Objective—T cell immunoglobulin and mucin domain (Tim) proteins are expressed by numerous immune cells, recognize phosphatidylserine on apoptotic cells, and function as costimulators or coinhibitors. Tim-1 is expressed by activated T cells but is also found on dendritic cells and B cells. Tim-4, present on macrophages and dendritic cells, plays a critical role in apoptotic cell clearance, regulates the number of phosphatidylserine-expressing activated T cells, and is genetically associated with low low-density lipoprotein and triglyceride levels. Because these functions of Tim-1 and Tim-4 could affect atherosclerosis, their modulation has potential therapeutic value in cardiovascular disease.

Approach and Results—ldlr−/− mice were fed a high-fat diet for 4 weeks while being treated with control (rat immunoglobulin G1) or anti-Tim-1 (3D10) or -Tim-4 (21H12) monoclonal antibodies that block phosphatidylserine recognition and phagocytosis. Both anti-Tim-1 and anti-Tim-4 treatments enhanced atherosclerosis by 45% compared with controls by impairment of efferocytosis and increasing aortic CD4+ T cells. Consistently, anti-Tim-4–treated mice showed increased percentages of activated T cells and late apoptotic cells in the circulation. Moreover, in vitro blockade of Tim-4 inhibited efferocytosis of oxidized low-density lipoprotein–induced apoptotic macrophages. Although anti-Tim-4 treatment increased T helper cell (Th)1 and Th2 responses, anti-Tim-1 induced Th2 responses but dramatically reduced the percentage of regulatory T cells. Finally, combined blockade of Tim-1 and Tim-4 increased atherosclerotic lesion size by 59%.

Conclusions—Blockade of Tim-4 aggravates atherosclerosis likely by prevention of phagocytosis of phosphatidylserine–expressing apoptotic cells and activated T cells by Tim-4–expressing cells, whereas Tim-1–associated effects on atherosclerosis are related to changes in Th1/Th2 balance and reduced circulating regulatory T cells. (Arterioscler Thromb Vasc Biol. 2016;36:456-465. DOI: 10.1161/ATVBAHA.115.306860.)

Key Words: apoptosis ◼ atherosclerosis ◼ inflammation ◼ macrophage ◼ T cells ◼ Tim

Cardiovascular disease is the leading cause of death worldwide and is mainly caused by atherosclerosis and its thrombotic complications. Atherosclerosis is characterized by the combination of lipid accumulation and inflammatory immune processes in the arterial wall. Current treatment options, primarily based on lipid-lowering, are inadequate to halt progression of cardiovascular disease with respect to lesion size or to reverse existing lesions, emphasizing an urgent need for new therapeutic strategies to inhibit atherosclerosis.

Apoptotic cell death is an important feature for maintenance of immune homeostasis in the atherosclerotic lesion. Early in the process of atherosclerotic lesion development, clearance of apoptotic cells, such as oxidized low-density lipoprotein (oxLDL)–loaded macrophages (foam cells), in the intima induces an anti-inflammatory response that limits lesion growth. However, in later stages of atherosclerosis, the clearance of apoptotic cells, or efferocytosis, is defective, causing secondary cellular necrosis that leads to the formation of necrotic cores and induces inflammatory responses that contribute to atherosclerosis progression.

Apoptotic cells can be recognized by different eat me signals, such as surface exposure of plasma membrane phosphatidylserine (PS), which facilitates uptake by phagocytes. T cell immunoglobulin and mucin domain (Tim) proteins are type 1 transmembrane proteins expressed on various immune cells that can recognize PS-exposing cells and can also mediate coinhibitory and costimulatory signals. Three TIM genes have been identified in humans (TIM-1, -3, and -4) and are associated with enhanced susceptibility to allergy and several autoimmune diseases, such as experimental autoimmune encephalomyelitis and diabetes mellitus. It has been shown that targeting of Tim-3 aggravates early and advanced...
Atherosclerosis in low-density lipoprotein receptor (ldlr)−/− mice, however, the role of Tim-1 and Tim-4 in atherosclerosis is not known.

Tim-1 is expressed on activated T cells and regulatory B cells, whereas Tim-4 expression is restricted to antigen-presenting cells. Tim-4 has particularly high expression on peritoneal macrophages and splenic marginal zone macrophages that are involved in maintaining immune homeostasis. Mice deficient for Tim-1 or Tim-4 have increased susceptibility for autoimmunity, as shown by increased T helper cell (Th)2 responses in tim-1−/− mice and hyperactive T and B cell responses in tim-4−/− mice. Recently, Lind et al found an association between Tim-1 and plaque occurrence in carotid arteries in a human population-based study using proteomic arrays. Interestingly, a single nuclear polymorphism in the Tim-4-encoding gene TIMD4 is associated with lowered LDL, triglycerides, and cardiovascular disease. However, it is not known whether this single nuclear polymorphism affects the expression or function of Tim-4. Another study showed that tim-4 mRNA negatively correlated with LDL levels in mice having type 2 diabetes mellitus.

Given their immunosuppressive potency by regulating clearance of PS-expressing cells, affecting adaptive immune responses and their possible association with LDL and triglycerides, modulation of Tim-1 and Tim-4 may represent a novel therapeutic target to treat cardiovascular disease. In the present study, we, therefore, investigated the role of Tim-1 and Tim-4 in atherosclerosis using blocking antibodies against Tim-1 and Tim-4.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Increased Percentages of Tim-1+ T Cells and Decreased Percentages of Tim-4+ Macrophages in Atherosclerotic Mice

Although Tim-1 is mainly expressed on T cells, Tim-4 expression is restricted to antigen-presenting cells and is highly expressed particularly on splenic marginal zone macrophages. To determine the percentage of Tim-1+ T cells in the spleen of atherosclerotic mice, we fed ldlr−/− mice a high-fat diet (HFD) for 0 (n=7), 4 (n=9), or 10 weeks (n=6). As shown in Figure 1A, CD3+CD4+Tim-1+ cells but not CD3+CD8+Tim-1+ cells were increased after 10 weeks of HFD. Moreover, Tim-1 was also expressed on splenic B cells, and CD19+Tim-1+ B cells were decreased after 10 weeks of HFD. Tim-4+ macrophages defined as F4/80+CD11c−Tim-4+ cells were decreased in spleens of mice fed a HFD for 10 weeks (Figure 1B). Because macrophages are abundantly present in atherosclerotic lesions, we analyzed Tim-4 expression on macrophages from aortic digests (Figure I in the online-only Data Supplement). Aortas were isolated from ldlr−/− mice fed a control or HFD for 10 weeks, and as shown in Figure 1C, the percentage Tim-4+ macrophages in the HFD group, which will include largely lesional macrophages, was 57.55%, slightly lower than the 66% Tim-4+ macrophages found in control diet group, which are mainly adventitial macrophages. Moreover, we measured Tim-4 expression on CD11c+MHCII+ dendritic cells and CD11b−B220+ B cells (Figure I in the online-only Data Supplement). As shown in Figure 1D, Tim-4 is also expressed on dendritic cells isolated from the aorta; however, the expression is not affected by HFD. We did not detect Tim-4 on B cells found in the aorta of either control or atherosclerotic mice (Figure 1E).

Blockade of Tim-4 Inhibits Uptake of oxLDL-Loaded Apoptotic Macrophages and Induces a Proinflammatory Phenotype

Tim-4+ macrophages are known to play an important anti-inflammatory role by engulfing PS-expressing apoptotic cells. In early atherosclerotic lesions, apoptotic oxLDL-loaded macrophage foam cells are efficiently cleared by neighboring phagocytes, but interference with this process can lead to necrotic death of foam cells, stimulating inflammatory responses by other macrophages. To investigate the importance of Tim-4 in this process, we isolated peritoneal macrophages and loaded them with an amount of oxLDL which causes macrophage apoptosis (100 μg/mL) for 48 hours (Figure 2A). Subsequently, we added these apoptotic macrophages to freshly isolated peritoneal macrophages that were preincubated for 30 minutes with 20 μg/mL rat immunoglobulin (Ig) G or anti-Tim-4 (21H12). As shown in Figure 2B, Tim-4 blockade significantly inhibits uptake of oxLDL-induced apoptotic macrophages by 69%. Furthermore, 24 hours after removing apoptotic macrophages from the coculture, we measured cytokine production in the supernatant of the phagocytes. Blockade of Tim-4 increased macrophage secretion of the proinflammatory cytokines interleukin (IL)-6 and monocyte chemotactant protein-1 (Figure 2C), suggesting that defective clearance of oxLDL-induced apoptotic macrophages mediated by Tim-4 blockade could have enhanced macrophage activation through the accumulation of necrotic cells or through oxidized phospholipids on the surface of the apoptotic cells.
To assess the role of Tim-1 and Tim-4 in atherosclerosis, we blocked these molecules with rat anti-mouse monoclonal antibodies in \(^{ldlr-/-}\) mice while they were fed a HFD for 4 weeks (Figure 3A). The 4-week end point was chosen because after this point, mouse anti-rat Ig responses would impair the effectiveness of the antibodies and lead to immune complex formation that could affect the disease process. At euthanasia, we did not observe any difference in body weight, or serum cholesterol and triglyceride levels were unaffected by blockade of Tim-1 or Tim-4 (Figure 3B; Figure IIA in the online-only Data Supplement). As shown in Figure 3C, lesion area as a percentage of the lumen was significantly higher (45% increase) in mice treated with anti-Tim-1 (7.76±0.78%) or anti-Tim-4 (7.76±0.67%) in comparison with control mice (5.37±0.37%; \(P<0.05\)). No significant difference in macrophage content (% lesion area) was observed between control mice (83.12±0.69%), anti-Tim-1-treated mice (81.02±2.38%), and anti-Tim-4-treated mice (80.89±1.63%, Figure 3D).

**Impaired Efferocytosis in Anti-Tim-4-Treated Mice**

Because Tim-1 and Tim-4 are involved in the recognition and removal of apoptotic cells, we first performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to detect apoptotic cells in atherosclerotic lesions. We observed a 3.2-fold increase in TUNEL+ cells in lesions of anti-Tim-1-treated mice (97.0±33.2 cells/mm²) and a significant increase (96.0±33.2 cells/mm²) in lesions of anti-Tim-4-treated mice (95.0±33.2 cells/mm²) in comparison with control mice (83.12±0.69%).

**Figure 2.** Blockade of Tim-4 inhibits uptake of oxidized low-density lipoprotein (oxLDL)-induced apoptotic macrophages and induces a proinflammatory phenotype. Macrophages isolated from the peritoneum of B6 mice (n=3) were exposed to 5, 50, or 100 \(\mu\)g/mL of oxLDL for 48 hours. The percentage of apoptotic cells (AnnexinV+/PI+ cells) was determined with flow cytometry (A). To assess whether Tim-4 blockade could interfere with uptake of oxLDL-induced apoptotic macrophages by macrophages, we first preincubated freshly isolated peritoneal macrophages with rat IgG or anti-Tim-4 (21H12, 20 \(\mu\)g/mL) for 30 minutes in triplicate. Subsequently, CFSE-labeled oxLDL-induced apoptotic macrophages were added in a 1:1 ratio, and after 3 hours, cells were stained for F4/80, and uptake of apoptotic cells was determined by immunohistochemistry (B). Cytokines (IL-6 and MCP-1) were measured in the supernatant of peritoneal macrophages preincubated with rat IgG (n=3) or anti-Tim-4 (21H12, n=3) 24 hours after a 3-hour incubation with oxLDL-induced apoptotic peritoneal macrophages (C). \(P<0.05\), \(***P<0.001\). IL indicates interleukin; MCP, monocyte chemoattractant protein; and Tim, T cell immunoglobulin and mucin domain.
4.3-fold increase in TUNEL + cells in lesions of anti-Tim-4-treated mice (129.1±22.6 cells/mm²) compared with control mice (30.0±18.5 cells/mm²; Figure 4A). Subsequently, we investigated the effect of Tim-1 and Tim-4 blockade on efferocytosis locally within the atherosclerotic lesion, as measured by the percentage of free TUNEL + cells versus macrophage-associated TUNEL + cells. As shown in Figure 4B, efferocytosis is strongly impaired by Tim-4 blockade and, although not significant, is also impaired in anti-Tim-1-treated mice. Moreover, we observed a defect in clearance of apoptotic cells in the circulation (Figure 4C) and more specifically of CD4 + T cells in anti-Tim-4-treated mice (Figure 4D).

Increased T Cell Numbers in the Aortic Root of Anti-Tim-1 and Anti-Tim-4-Treated Mice

Previously, it has been described that Tim-4-expressing macrophages can engulf activated T cells that express PS at their surface shortly after activation. In line with this finding, we observed increased circulating CD4 +CD69 + T cells in anti-Tim-4-treated mice (6.47±0.54%) compared with control mice (4.83±0.34%; Figure 4E). Because both Tim-1 and Tim-4 have been implicated in T cell function and survival, we investigated whether blockade of these molecules affects T cell numbers in atherosclerotic lesions of ldlr −/− mice. Representative cross-sections of the aortic root were stained for CD4 and CD8 (Figure 4F, red cells). Treatment with anti-Tim-1 significantly enhanced CD4 + T cell numbers within the lesions (10.25±1.06 cells; P<0.05) in comparison with control mice (6.61±1.11 cells), whereas numbers of CD4 + T cells in the surrounding perivascular tissue were elevated in both anti-Tim-1 (13.53±1.59 cells; P<0.05) and anti-Tim-4-treated mice (15.68±1.75 cells; P<0.01) in comparison with control mice (7.55±1.29 cells). In contrast, we did not observe significant differences between groups in the number of CD8 + T cells in atherosclerotic lesions or in the perivascular tissue (Figure IIB in the online-only Data Supplement).

Blockade of Tim-1 and Tim-4 Affect Different Subsets of Effector T Cells

Tim-4 expression on marginal zone macrophages in the spleen is essential for the maintenance of immune homeostasis by clearance of apoptotic cells and regulation of the number of activated T cells. Blockade of Tim-4 inhibits clearance of these cells and, thereby, promotes inflammation. Consistent with this, we found slightly enlarged spleens and an increased percentage of proliferating CD4 +Ki-67 + cells...
in anti-Tim-4-treated mice in comparison with control mice (Figure 5A). However, the percentage of CD4+ and CD8+ T cells in the spleen did not differ (Figure IIC in the online-only Data Supplement).

To evaluate whether blockade of Tim-1 and Tim-4 affects specific effector T cell subsets, at euthanasia, splenocytes were stimulated with phorbol 12-myristate 13-acetate/ionomycin/Brefeldin A for 4 hours and stained for interferon-γ (IFNγ)-secreting CD4+ cells (Th1 cells), IL-4-secreting CD4+ cells (Th2 cells), and IL-17-secreting CD4+ cells (Th17 cells; Figure 5B). The majority of the pathogenic CD4+ T cells in atherosclerosis are Th1 cells. Mice treated with anti-Tim-4 showed elevated Th1 cells (5.37±0.51%) in comparison with control mice (2.32±0.37%, P<0.01). Although blockade of Tim-1 did not affect the percentage of Th1 cells (2.84±0.12%), Th2 cells were strongly increased (0.71±0.06%) in comparison with control mice (0.31±0.11%; P<0.05). The percentage of Th2 cells in anti-Tim-4-treated mice (0.59±0.09%) was also increased (albeit not significantly) compared with control mice. We did not detect any Th17 cells by flow cytometry.

To determine whether blockade of Tim-1 and Tim-4 affects regulatory T cells (Tregs), we stained blood for CD4, CD25, and the transcription factor Foxp3. We detected a significant 76% reduction of circulating Tregs in Tim-1-treated mice (0.46±0.21%) compared with control mice (1.90±0.22%; P<0.001). In line with our flow cytometry data, Th2 cytokines were increased in the supernatants of anti-CD3/CD28-stimulated splenocytes of anti-Tim-1-treated mice (IL-4 and IL-13), whereas supernatant of splenocytes from anti-Tim-4-treated mice contained high levels of both Th1 and Th2 cytokines (IFNγ, IL-4, and IL-13; Figure 5C). Moreover, IL-6 and IL-17 levels were significantly increased in cultures from anti-Tim-4-treated mice in comparison with control mice.

Humoral Responses in Anti-Tim-1 and Anti-Tim-4-Treated Mice

Blockade of Tim-1 or Tim-4 during atherosclerosis strongly induced Th2 and Th1/Th2 responses, respectively. Because isotype switching of B cells is dependent on cytokine secretion by T cells, we investigated whether B cell antibody production was affected in control, anti-Tim-1-, and anti-Tim-4-treated mice after 4 weeks of treatment and HFD. IL-4 secretion can induce an IgG1 isotype switch, whereas IFNγ can induce an IgG2c switch. Accordingly, Figure 5D shows a significant increase in total IgG2c and IgG1 in serum of anti-Tim-4-treated mice in comparison with control mice. Both anti-Tim-1 and anti-Tim-4
treatments induced IgG1 antibodies against oxLDL and malondialdehyde-modified low-density lipoprotein (MDA-LDL) in comparison with control treatment. Notably, IgM antibodies against oxLDL and MDA-LDL, as well as the IgM Nab E06, were also elevated after Tim-1 and Tim-4 blockade (Figure II in the online-only Data Supplement).

**Tim-1 and Tim-4 Act Nonredundantly in Aggravating Atherosclerosis**

Because both Tim-1 and Tim-4 are important regulators of immune homeostasis and have been described to interact with each other, but also have distinct functions, we were interested to determine whether blockade of both Tim-1 and Tim-4 at the same time would have a greater effect than either alone on atherosclerosis. To assess the role of combined blockade of Tim-1 and Tim-4 in atherosclerosis, we treated LDLr<sup>−/−</sup> mice twice weekly intraperitoneally with 200 μg of anti-Tim-1 and 200 μg anti-Tim-4 or 400 μg of the isotype control (rat IgG1), while the mice were fed a HFD for 4 weeks (Figure 6A). At euthanasia, we did not observe any difference in body weight or serum cholesterol levels (Figure 6B; Figure IIIA in the online-only Data Supplement). Interestingly, triglyceride levels were decreased in anti-Tim-1+4-treated mice (200.30±15.34 mg/dL) in comparison with control mice (279.40±26.64 mg/dL; P<0.05). Lesion area was 59% greater in mice with combined blockade of Tim-1 and Tim-4 (9.12±1.12%) in comparison with control mice (5.75±1.02%; P<0.05; Figure 6C). No significant difference in lesional macrophage content was observed (control: 77.06±2.32% versus anti-Tim-1+4: 78.05±2.52%; Figure 6D).

Moreover, treatment with anti-Tim-1+4 significantly enhanced CD4<sup>+</sup> T cell numbers within the lesions (11.00±1.75...
cells; P<0.05) and in perivascular tissue (9.56±1.28 cells; P<0.05) in comparison with control mice (5.80±1.16 cells and 5.30±1.28 cells, respectively; Figure 6E; Figure IIIB in the online-only Data Supplement), whereas CD8+ T cells were unaffected (Figure IIIC in the online-only Data Supplement). Simultaneous blockade of Tim-1 and Tim-4 results in a 7.8-fold increase in TUNEL+ cells (154.3±27.7 cells/mm² versus 19.7±8.5 cells/mm² in control mice; Figure 6F). This increase in CD4+ T cells in anti-Tim-1+4-treated mice corresponds to impaired clearance of apoptotic T cells (Figure IIID in the online-only Data Supplement) and is consistent with enlarged spleens, increased spleen cellularity, and enhanced proliferation in these mice (Figure IIIE in the online-only Data Supplement). Combined blockade of Tim-1 and Tim-4 also increased inflammatory CD11b+Ly6G−Ly6Chi monocytes in the spleen (Figure IIIF in the online-only Data Supplement), whereas there is no difference in blood (data not shown). Furthermore, circulating Tregs were decreased in anti-Tim-1+4-treated mice (Figure 6G). Surprisingly, both Th1 and Th2 cytokine secretion (IFNγ and IL-4) was reduced after treatment with anti-Tim-1+4 (Figure 6H), whereas similar to our atherosclerosis experiments with individual Tim-1 and Tim-4 treatments, total IgG1 and IgG1 specific for oxLDL and MDA-LDL were increased in anti-Tim-1+4-treated mice (Figure 6I;
Figure IV in the online-only Data Supplement). Additionally, oxLDL-specific IgG2c and total IgM was increased after combined blockade of Tim-1 and Tim-4. We also did not observe a difference in the total percentage of CD19+ B cells or B cell subsets as defined by CD19+CD3 IgM+IgD− and CD19+CD3 IgM+IgD− cells (Figure IVE in the online-only Data Supplement).

Discussion

Tim-1 and Tim-4 have diverse and partly overlapping functions related to regulation of inflammatory and immune responses, including T cell costimulation, and clearance of apoptotic cells. Some of the functions of Tim-1 and Tim-4 suggest that they may be useful targets for therapy of immunologic diseases. Given the central role of innate and adaptive immunity and clearance of apoptotic cells in atherosclerotic lesion development and phenotype, it is likely that Tim-1 and Tim-4 influence this disease process. Nonetheless, the net effect of blockade of either Tim-1 or Tim-4 on atherosclerosis is not predictable from prior studies of the functions of these molecules in other disease contexts. We, therefore, determined how atherosclerosis is affected in mice treated with blocking antibodies, modeling the hypothetical treatment of humans with similar reagents.

In this study, we demonstrate that treatment with anti-Tim-1 (3D10) or anti-Tim-4 (21H12) aggravates atherosclerosis, independent of cholesterol and triglyceride levels. Two important processes contributing to atherosclerosis development were affected by Tim-1 or Tim-4 blockade: efferocytosis and adaptive immune responses.

Previously, it has been shown that peritoneal macrophages and B1 cells from TIM-4−/− mice do not clear apoptotic bodies in vivo. In addition, studies using anti-Tim-4 (21H12) have shown that blockade of Tim-4 reduces phagocytosis of apoptotic cells by peritoneal or splenic marginal zone macrophages. In line with these findings, we observed that Tim-4 blockade with 21H12 potently inhibits uptake of oxLDL-induced apoptotic macrophages by peritoneal macrophages in vitro and induces secretion of proinflammatory cytokines. Consistently, anti-Tim-4–treated mice have increased percentages of circulating late apoptotic cells, indicative of impaired efferocytosis that contributes to lesion growth. Interestingly, a recent paper describes a subset of tissue-resident Tim-4+CD169+ that are immunoregulatory by inducing Tregs, reducing T cell proliferation, and promoting a higher rate of death of activated T cells. These Tim-4+CD169+ cells are highly susceptible for apoptosis, and if Tim-4+CD169+ cells would not undergo apoptosis when Tim-4 is blocked, we predict we would have observed reduced apoptosis in our experiment. However, it has been shown that depletion of CD169+ macrophages increases necrotic core size and apoptotic cell content of atherosclerotic lesions, and also in our study, we observed a significant increase in apoptotic TUNEL+ cells in lesions of anti-Tim-4–treated mice and show that Tim-4 blockade impairs efferocytosis. This indicates that the macrophages in the absence of Tim-4 are not able to recognize PS-expressing apoptotic cell, resulting in accumulation of apoptotic cells, which undergo secondary necrosis and trigger inflammation. Additionally, we also observe a trend toward increased apoptotic cells in atherosclerotic lesions of anti-Tim-1–treated mice. Although Tim-1 is not expressed on macrophages, it has been shown that Tim-1 can be present on CD11c+ dendritic cells and on B cells, which both have the ability to phagocytize apoptotic cells and apoptotic bodies. Xiao et al have shown previously that B cells of Tim-1–deficient mice showed a defect in binding and uptake of apoptotic cells. It is, therefore, possible that the increase in numbers of TUNEL+ cells in atherosclerotic lesions of anti-Tim-1–treated mice is a consequence of impaired uptake of apoptotic cells by Tim-1+ B cells. Moreover, there is a certain subset of CD11c+ cells that coexpress F4/80, which may explain the observed impaired efferocytosis capacity of F4/80+ cells in anti-Tim-1–treated mice.

Previous studies have shown that splenocyte cultures from mice immunized with apoptotic cells spontaneously release high levels of Th1 and Th2 cytokines. Moreover, Tim-1−/− mice and Tim-4−/− mice have hyperactive T and B cells, as shown by increased proliferation, increased IFNγ and IL-17 secretion and elevated circulating immunoglobulins, whereas Tim-4 Tg mice have reduced memory T cell responses, and their T cells do not produce IL-4 or IFNγ. In our study, we show that blockade of either Tim-1 or Tim-4 increased the number of lesional or perivascular CD4+ T cells. Although anti-Tim-4 treatment increased splenic IFNγ-secreting Th1 cells and IL-4-secreting Th2 cells, anti-Tim-1 treatment only induced Th2 cells. IL-17 secretion was also increased by anti-CD3/CD28-stimulated splenocytes from anti-Tim-1 or anti-Tim-4–treated mice. In atherosclerosis, it has been well established that Th1 cells are proatherogenic, and some studies show that IL-4-deficient ldlr−/− and apoe−/− mice have reduced atherosclerosis.

The role of IL-17 in atherosclerosis remains controversial but exogenous IL-17 administration has been shown to promote the formation of atherosclerotic lesions. In addition to increased Th2 responses, anti-Tim-1–treated mice had strongly reduced circulating Tregs which exert a protective role in atherosclerosis. Consistent with our findings that Tim-1 and Tim-4 are atheroprotective by their effects on T cell responses, Xiao et al show that Tim-1–deficient B cells promote Th1 and Th17 cells, inhibit Tregs, and enhance the severity of experimental autoimmune encephalomyelitis. Blockade of Tim-4 with RMT4-53 exacerbates the induction phase of collagen-induced arthritis by enhanced T cell proliferation and increased IFNγ and IL-17 secretion. Furthermore, Albacker et al showed that Tim-4 blockade (21H12) reversed the induction of intranasal tolerance, resulting in increased proliferative and cytokine responses (IL-4, IFNγ) of T cells. In addition, reduced CD4 T cell responses in Tim-4–treated mice lead to reduced airway hyper-responsiveness in a murine model of asthma.

Previously, it has been shown that T cells also express PS shortly after activation, and anti-Tim-4 (21H12) treatment was shown to specifically reduce phagocytosis of antigen-specific T cells. Indeed, anti-Tim-4–treated ldlr−/− mice had elevated levels of circulating activated T cells, and their T cells showed enhanced proliferative capacity, which may have contributed to enhanced aortic T cells.
Moreover, immunoglobulin levels associated with Th1 and Th2 responses, IgG2c and IgG1, respectively, were elevated in anti-Tim-1 or anti-Tim-4-treated mice. Interestingly, compared with control mice, we also observed increased IgM levels against oxLDL and MDA-LDL in the anti-Tim-1 or anti-Tim-4 mice, as well as an increase in the E06 NAb against phosphocholine of oxPL, as found on oxLDL. It is notable that apoptotic cells express increased epitopes of both MDA and oxPL. Both enhanced atherosclerosis and enhanced content of apoptotic cells secondary to anti-Tim-1 and anti-Tim-4 could have provided the antigen exposure to increase titers of these IgM antibodies. It has previously been shown that these specific IgMs have anti-atherosclerotic potential because of their ability to enhance clearance of apoptotic cells and their inhibitory effect on foam cell formation. Despite this atheroprotective effect, we observe enhanced atherosclerosis in anti-Tim-1 or anti-Tim-4-treated mice. Previously, it has been shown that mice immunized with apoptotic cells develop high IgM and IgG titers to oxidation-specific epitopes of oxLDL which contributes to their clearance. Thus, although the titers of these IgM antibodies were increased, presumably they were insufficient to fully compensate for the increased apoptotic cell number and enhanced macrophage foam cell generation. Moreover, the skewing toward proatherogenic T cell responses could have dominated over an atheroprotective effect of increased IgM.

Moreover, we show that treatment of ldlr<sup>−/−</sup> mice with anti-Tim-1 and anti-Tim-4 does not affect the total percentage of CD19<sup>+</sup> B cells and does not affect B cell subsets as defined by CD19<sup>+</sup>CD3<sup>+</sup>IgM<sup>high</sup>IgD<sup>low</sup> and CD19<sup>+</sup>CD3<sup>+</sup>IgM<sup>low</sup>IgD<sup>high</sup> cells. It has been described that Tim-1 is expressed by a subset of IL-10-producing B cells: Bregs. However, it recently has been shown that IL-10-producing B cells, or Bregs, are dispensable for atherosclerosis development in mice. It is, however, possible that reduced Bregs affect the Treg population and thereby indirectly affect atherosclerosis. Further research will clarify the importance of Tim-1<sup>+</sup> Bregs in atherosclerosis.

Other than binding to PS and blocking apoptotic cell clearance, Tim-1 and Tim-4 have many nonoverlapping functions and are expressed on different cell types. Therefore, we reasoned that the proatherogenic effects of blocking both molecules would be greater than blocking only one. Indeed, we found that combined blockade had a greater effect on lesion development than each single molecule blockade, and this correlated with more robust effects on apoptotic cell clearance, lesional T cell numbers, and serum anti-oxLDL antibodies. In addition, combined blockade of Tim-1 and Tim-4 increased inflammatory CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>+</sup> monocytes in the spleen. Previously, Robbins et al have shown that Ly6C<sup>+</sup> monocytes that originate from the spleen accumulate in the arterial wall and enhance atherosclerotic lesion formation. Because we did not detect Tim-1 and Tim-4 expression on monocytes (unpublished data), the increase in monocytes on Tim-1 and Tim-4 blockade is likely caused by enhanced inflammation following impaired clearance of apoptotic cells and changes in Th1/Th2 cell ratios.

In summary, treatment with a blocking Tim-4 antibody aggravates atherosclerosis by prevention of phagocytosis of PS-expressing apoptotic cells and activated T cells by Tim-4-expressing cells, whereas Tim-1-associated effects on atherosclerosis are related to changes in Th1/Th2 balance and reduced circulating Tregs. Future experiments focusing on stimulation of Tim-1 or Tim-4 signaling may lead to new therapeutic insights for the prevention of cardiovascular diseases.

Sources of Funding

This work was supported by National Institutes of Health grant HL087282 (A.H. Lichtman), P01AI054456 and P01AI089955 (G.J. Freeman and R.H. DeKruyff) and a fellowship from the International Atherosclerosis Society and the Young Talent Program of the GENIUS consortium (CVON, Dutch Heart Foundation; A.C. Foks).

Disclosures

None.

References


Significance

Myocardial infarction and stroke are the leading cause of mortality worldwide and are generally triggered by rupture of an atherosclerotic plaque. Current treatment consisting of statins and lifestyle advice is inadequate to halt progression or induce regression of atherosclerotic lesions, indicating an urgent need for new therapeutic strategies to inhibit atherosclerosis. Transmembrane T cell immunoglobulin and mucin domain proteins are expressed on various immune cells and exert functions related to regulation of inflammatory and immune responses, including T cell costimulation, and clearance of apoptotic cells, which are processes of known importance in atherosclerosis. Therefore, we determined how atherosclerosis is affected in mice treated with blocking antibodies, modeling the hypothetical treatment of humans with similar reagents. We show that blockade of T cell immunoglobulin and mucin domain-1 and -4 aggravates atherosclerosis, suggesting that stimulating T cell immunoglobulin and mucin domain signaling might be protective and could be of therapeutic value in cardiovascular disease.
Blockade of Tim-1 and Tim-4 Enhances Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice
Materials and Methods

Animals
Female and male ldlr deficient (ldlr−/−) mice, 8-10 weeks old, were obtained from Jackson Laboratories. The animals were fed a regular chow diet or chemically defined cholesterol containing high-fat diet (HFD) (Research Diets Inc, New Brunswick, NJ, USA). Diet and water were provided ad libitum. All mice were maintained in a pathogen-free facility at the Warren Alpert Building of Harvard Medical School in accordance with the animal research guidelines established by the Committee of Animal Research and the National Institutes of Health.

Coculture of apoptotic macrophages and fresh macrophages
Resident peritoneal cells were isolated from B6 mice and plated in a 10ml petri dish for 2 hours at 37°C for adherence of macrophages. Macrophages were detached from the petri dish with EDTA and cultured at 1x10^6 cells/ml in the presence of oxLDL (100μg/ml) for 48 hours. Subsequently, the apoptotic cells were washed, labeled with CFSE, using the Vybrant CFDA-SE Cell Tracer Kit according to manufacturer’s protocol (Invitrogen), and added to freshly-isolated peritoneal macrophages that were preincubated for 30 minutes with 20μg/ml rat IgG or rat anti-Tim-4 IgG (21H12). After 3 hours, cells were washed and uptake of apoptotic cells by macrophages (F4/80+ cells, stained using Alexa Fluor 647, clone BM8, diluted 1:100) was assessed using a Nikon Eclipse TI fluorescence microscope and Metamoph software. In a separate experiment, peritoneal macrophages preincubated with 20μg/ml rat IgG or rat anti-Tim-4 IgG (21H12), were cocultured with oxLDL-induced apoptotic cells for 3 hours. Subsequently, unbound apoptotic cells were washed away and macrophages were kept in culture for an additional 24 hours after which supernatant was collected to measure cytokines using a Luminex bead-based multiplex assay.

Tim-1 and Tim-4 blockade
Atherosclerosis was induced in ldlr−/− mice by feeding mice a HFD for 4 weeks. During these 4 weeks, mice received twice weekly i.p. injections of 200μg anti-Tim-1 (clone 3D10, n=14), 200μg anti-Tim-4 (clone 21H12, n=20) or 200μg of an isotype control (rat IgG1, n=19). In separate experiments, combined blockade of Tim-1 and Tim-4 was performed by injecting mice twice weekly i.p. with 200μg anti-Tim-1 and 200μg anti-Tim-4 (n=10) or 400μg of the isotype control (rat IgG1, n=10). After 4 weeks, mice were sacrificed and tissues were harvested after in situ perfusion using PBS. Tissues were stored in 4% PFA or snap frozen in nitrogen and stored at -80°C until further use.

Serum lipids
At sacrifice, mice were weighed and plasma was collected via heart puncture. Lipid analyses were performed on the cobas c 501 analyzer (Roche Diagnostics, Indianapolis, IN) using clinical assays developed for human specimens. Results of these assays, when performed in mouse specimens, correlate well with results obtained using alternative tests. Total cholesterol concentration was measured using the CHOL2 assay, HDL cholesterol was measured using the HLDC3 assay, LDL cholesterol was measured directly using the LDL_C assay and triglyceride levels were measured using the TRIGL assay (all Roche Diagnostics).

Histological analysis and morphometric analysis of aortic atherosclerosis
Analysis of atherosclerotic lesion size was performed on 5 Oil-red-O-stained cryosections (10μm each) spanning 160 μm of the three-valve area of the aortic root. Furthermore, sections were stained immunohistochemically with an antibody directed against CD4 T cells (clone RM4-
5 BD Pharmingen, diluted 1:90) and CD8 T cells (clone Ly-2 BD Pharmingen, diluted 1:100). Biotinylated rabbit anti-rat IgG (Vector, dilution 1:200) was used as a secondary antibody and following incubation with Streptavidin-HRP (K0675 DAKO) the reaction was visualized with 3-amino-9-ethyl carbazole (AEC, K3464 DAKO). Furthermore, corresponding sections were stained immunohistochemically with an antibody directed against a macrophage-specific antigen (Moma-2, monoclonal rat IgG2b, diluted 1:500). 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. Images of sections were photographed using a Nikon microphoto-FXA microscope and lesion area, or macrophage stained area were quantified using Image Pro Plus software. T cell numbers were manually counted. Apoptotic cells in lesions were stained using the in situ cell death detection kit (11684817910, Roche) according to manufacturer’s protocol. TUNEL positive nuclei were counted manually. In situ efferocytosis was determined by staining for apoptotic cells as described above and macrophages (rat anti-mouse F4/80, mca497GA Biorad) following an incubation with goat anti-rat IgG AF467 (A-21247 Life Technologies). Nuclei were stained with DAPI. Efferocytosis was assessed by counting the number of free vs. macrophage-associated apoptotic cells in individual lesion sections. Apoptotic cells were considered ‘free’ when they were not surrounded by or in contact with macrophages. Analysis was performed using an Olympus Fluoview1000.

Preparation of cells from lymphoid organs and aorta for flow cytometry
Spleens or lymph nodes were isolated and single cell suspensions were obtained by squeezing the spleens through a 70µm cell strainer. Red blood cells were removed using ACK lysing buffer (A10492-01, Gibco). Different immune cells were analyzed with flow cytometry: Aortas from the ascending aorta to iliac bifurcation were cleaned of peri-advential connective tissue and subjected to enzymatic digestion, as described.3 In brief, aortas were minced and subsequently digested with 450U/ml collagenase I-S (C1639, Sigma), 125U/ml collagenase XI (C7657, Sigma), 60U/ml hyaluronidase (H3506, Sigma) and 60U/ml DNase1 (D5025, Sigma) for 1 hour. Cells were washed with PBS twice and subsequently stained and analyzed by flow cytometry.

Flow cytometric analyses
Cells were analyzed by flow cytometry using the following antibodies (from Biolegend unless indicated): CD45.2 (104), CD11b (M1/70), B220 (RA3-6B2), CD11c (N418), MHCII (M5/114.15.2), Tim-1 (RMT1-4), Tim-4 (RMT4-54), Ki-67 (16A8), CD3 (17A2), CD4 (RM4-5), CD8 (Ly-2, BD Biosciences), CD69 (H1.2F3), Live/Dead Viability staining (Life Technologies) or 7-AAD (BD Pharmingen) was used for exclusion of non-viable cells. To investigate Th1 (CD4+IFNγ), Th2 (CD4+IL-4) and Th17 cells (CD4+IL-17+), splenocytes were stimulated with PMA (1µg/ml) and ionomycin (10ng) for 4 hours at 37°C. Brefeldin A (BD Biosciences) was added to the culture to enable measurement of intracellular cytokines. To detect Th1/Th2/Th17 and Treg cells (CD4+CD25+Foxp3+), cells were fixed and permeabilized according to manufacturer’s protocol (BD Biosciences). Subsequently, the cells were stained for IFNγ (XMG1.2, BD Biosciences), IL-4 (11B11, BD Biosciences), IL-17 (TC11-18H10.1, Biolegend), and Foxp3 (FJK-16a, eBioscience). To detect apoptotic cells, an Annexin-V/PI staining was performed on splenocytes according to manufacturer’s protocol (88-8007, eBioscience). FACS analysis was performed on a FACSCalibur (BD Biosciences) and a DXP12 Analyzer (Cytek). Data were analyzed using FlowJo software (Treestar).

Spleen cell proliferation
At sacrifice, splenocytes (n=5 per group) were cultured for 72 hours in triplicate in a 96 well round-bottom plate (2×10⁵ cells/well) in RPMI 1640 supplemented with L-Glutamine, 100 U/ml streptomycin/penicillin and 10% FCS. Cells were activated with anti-CD3 (1.25µg/ml, clone 145-
2C11) and anti-CD28 (1.25μg/ml, clone 37.51 Bioexpress) for 72 hours. Proliferation was assessed by manually counting cells.

**Multiplex cytokine assay**
Culture supernatants were analyzed for cytokine concentrations using a Luminex bead-based multiplex assay specific for IL-1α, IL-β, IL-2, IL-4, IL-5, IL-6, IL-10, 12p40, 12p70, IL-13, IL-17, IL-33, IFNγ, TNFα, MCP-1 and GM-CSF. Recombinant cytokine standards (Bio-Rad, Hercules, CA) were used to calculate cytokine concentrations and data were analyzed using StarStation 2.3 software (Applied Cytometry, Sheffield UK).

**Immunoglobulin (Ig) measurement**
Isotype- and antigen-specific antibody titers were determined by chemiluminescent enzyme immunoassays as previously described. In brief, antigens were coated at 5 μg/mL PBS overnight at 4°C (IgM (Rat a-ms IgM BD553435), IgG1 (goat anti-mouse-IgG1 (Jax115-005-205), IgG2c (Rat anti-mouse-IgG2 (BD 553446), AB1-2 (an anti-idiotype antibody specific for the T15/E06 Natural antibody), CuOxLDL, MDA-hLDL). The plates were blocked with 1% BSA in TBS, serially diluted antisera from individual mice were added and incubated for 1.5h at room temperature. Bound plasma immunoglobulin (Ig) isotype levels were detected with various anti-mouse Ig isotype-specific alkaline phosphatase (AP) conjugates (IgM: Abcam 99589, IgG1: Jackson 115-055-205, IgG2c: Jackson 115-055-208) using LumiPhos 530 (Lumigen, Southfield, MI, USA) solution, and a Dynex Luminometer (Dynex Technologies, Chantilly, VA, USA). Data are expressed as relative light units counted per 100 milliseconds (RLU/100 ms).

**Statistical analysis**
All data are expressed as mean±SEM. The shown n-values represent number of animals. An unpaired two-tailed Student T-test was used to compare normally distributed data between two groups of animals. A one-way ANOVA with Tukey's Multiple Comparison post-test was performed for three group experiments. Probability values of \( P<0.05 \) are considered significant.

**References**

SUPPLEMENTAL MATERIAL
Supplemental Figure I. Flow cytometric gating strategy for identification of Tim-4+ macrophages, dendritic cells and B cells in aortic digests of ldlr−/− mice fed a HFD. Pre-gating was done on live CD45+ cells.
Supplemental Figure II. Enhanced numbers of splenocytes and IgM levels in anti-Tim-1 or anti-Tim-4 treated mice. At sacrifice, serum LDL and HDL levels were determined in control (n=19), anti-Tim-1-treated (n=14) and anti-Tim-4-treated mice (n=20) (A). Representative cross-sections of lesions in the aortic valve area were stained for CD8+ T cells (bar=100 μm) and the number of T cells in the intima and perivascular tissue were manually analyzed (B). The percentage of CD4+ and CD8+ T cells in the spleen were measured by FACS (C). MDA-LDL-specific and oxLDL-specific IgG2c, total IgM and MDA-LDL-specific, oxLDL-specific, and EO6-specific IgM were detected in serum of control (n=19), anti-Tim-1-treated (n=14) and anti-Tim-4-treated mice (n=20) (D). *P<0.05, **P<0.01
Supplemental Figure III. Combined blockade of Tim-1 and Tim-4 affects T cell responses. At sacrifice, serum LDL, HDL and triglyceride levels were determined in control (n=10) and anti-Tim-1+4-treated mice (n=10) (A). The number of CD4⁺ T cells in the perivascular tissue were manually analyzed (B). Representative cross-sections of lesions in the aortic valve area of control (n=7) and anti-Tim-1+4-treated mice (n=9) were stained for CD8⁺ T cells (bars=100 μm) and the number of T cells in the intima and perivascular tissue were manually analyzed (C). At sacrifice, blood was isolated and the percentage of apoptotic cells was determined with an Annexin-V/PI staining and analyzed with flow cytometry (D). At sacrifice, spleens were weighed and the amount of splenocytes were determined (E). Proliferation of anti-CD3/CD28-stimulated splenocytes (n=5 per group) was determined by cell counts after 72 hours. At sacrifice, the percentage of Ly6C⁺ monocytes in the spleen of control (n=10) and anti-Tim-1+4-treated mice (n=10) was determined with flow cytometry (F). *P<0.05, **P<0.01, ***P<0.001
Supplemental Figure IV. Elevated immunoglobulin levels by combined blockade of Tim-1 and Tim-4. Total IgG2c and MDA-LDL-specific IgG2c (A), total IgG1 and MDA-LDL-specific IgG1 (B), and total IgM, oxLDL-specific IgM and MDA-LDL-specific IgM (C) were detected in serum of control (n=10) and anti-Tim-1+anti-Tim-4 treated mice (n=10). At sacrifice, CD19⁺CD3⁻ cells were determined in the spleen using flow cytometry (D). B cell subsets were assessed using IgD and IgM, distinguishing IgM<sub>int/low</sub>IgD<sub>hi</sub> B cells, IgM<sub>hi</sub>IgD<sub>low</sub> B cells, and IgM<sub>hi</sub>IgD<sub>hi</sub> B cells (E). *P<0.05, **P<0.01, ***P<0.001.