CD40 Ligand Mediates Inflammation Independently of CD40 by Interaction With Mac-1

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Background—Strong evidence supports a role for CD40 ligand (CD40L) as marker and mediator of inflammatory diseases such as atherosclerosis. Despite extensive characterization of CD40, the classic receptor of CD40L, its role in immune defense against inflammatory diseases remains uncertain. The present study aimed to characterize the contribution of CD40 signaling to atherogenesis.

Methods and Results—Surprisingly, mice deficient in both CD40 and the low-density lipoprotein receptor did not develop smaller lesions in the aortic arch, root, and thoracoabdominal aorta compared with mice deficient only in the low-density lipoprotein receptor that consumed an atherogenic diet for 8 and 16 weeks. By flow cytometry, radioactive binding assays, and immunoprecipitation, we demonstrate that CD40L interacts with the integrin Mac-1, which results in Mac-1–dependent adhesion and migration of inflammatory cells as well as myeloperoxidase release in vitro. Furthermore, mice deficient in CD40L show significantly reduced thioglycolate-elicited invasion of inflammatory cells into the peritoneal cavity compared with mice deficient in CD40 and wild-type controls. Inhibition of Mac-1 in low-density lipoprotein receptor–deficient mice attenuates lesion development and reduces lesional macrophage accumulation.

Conclusions—These observations identify the interaction of CD40L and Mac-1 as an alternative pathway for CD40L-mediated inflammation. This novel mechanism expands understanding of inflammatory signaling during atherogenesis. (Circulation. 2007;115:1571-1580.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ leukocytes ■ CD40 ligand ■ mice

CD40 ligand (CD40L; CD154), a 39-kD transmembrane protein that belongs to the tumor necrosis factor superfamily, first described on T lymphocytes, interacts with its receptor CD40, a 50-kD protein, first discovered on B cells. CD40/CD40L interactions participate in pivotal functions of T-cell–dependent humoral immunity.1 Recent reports have implicated both receptor and ligand with several inflammatory diseases such as atherosclerosis.2,3

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Cell types typically resident in atherosclerotic plaques such as endothelial cells, macrophages, and smooth muscle cells can express CD40 and CD40L.4,5 Exposure to CD40L stimulates a broad inflammatory response in these cells such as heightened expression of proinflammatory cytokines, adhesion molecules, matrix degrading enzymes, and procoagulants—all probable participants in atherogenesis and lesion complication.6 Indeed, we and others previously demonstrated that atherosclerosis-prone mice such as low-density lipoprotein receptor (LDLR)– or apolipoprotein E–deficient animals that lack CD40L genetically or those treated with neutralizing anti-CD40L antibodies develop markedly reduced levels of atherosclerosis on high-cholesterol diets.7,8 Similarly, atherosclerotic lesions in such animals display features associated with plaque stability (ie, reduced macrophage and lipid content as well as increased collagen con-
tent). Indeed, CD40L inhibition even retards progression of preexisting atherosclerotic lesions in such animals.\(^{9,10}\) Finally, soluble CD40L (sCD40L), the 18-kD soluble form predominantly released from platelets on platelet activation may identify first or recurrent cardiovascular events, which further supports the pathogenic role of CD40L.\(^{28–31}\)

The present study originally aimed to identify the contribution of the receptor CD40 to atherogenesis in mice. Unexpectedly, the results revealed that modulation of murine atherosclerosis by CD40L does not depend on its classic receptor, CD40. Therefore, we sought a potential alternative receptor for CD40L. CD40L binds to the platelet integrin glycoprotein (GP) Ibβ/IIa (\(\alpha_{\text{IIb}}\gamma_3\), CD41/CD61) and induces outside-in signaling and thereby platelet activation.\(^{14,15}\) The major ligand of GP Ibβ/IIa is the plasma protein fibrinogen. However, fibrinogen can also bind another integrin receptor, Mac-1 (\(\alpha_{\text{M}}\beta_2\), CD11b/CD18), expressed abundantly on monocytes/macrophages, a cell type of pivotal importance in atherogenesis. Mac-1 function is strongly and rapidly regulated via conformational changes of the receptor, which is also true for GP Ibβ/IIa. Mac-1 is a highly promiscuous receptor that interacts with ligands such as C3bi,\(^{16}\) intracellular adhesion molecule-1,\(^{17}\) fibrinogen,\(^{18}\) vitronectin,\(^{19}\) factor Xa,\(^{20,21}\) heparin,\(^{22,23}\) GP Ib\(\alpha\),\(^{24,25}\) JAM-3,\(^{26}\) and lipoprotein (a).\(^{27}\) Based on these interactions, Mac-1 participates in processes important in atherosclerosis such as inflammation, immune response, and coagulation.\(^{28–31}\) Therefore, we further tested the hypothesis that CD40L interacts with the monocyte/macrophage integrin Mac-1.

### Methods

A detailed description of all methods is accessible in the online Data Supplement. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

### Results

#### CD40-Deficiency Does Not Attenuate the Development of Atherosclerotic Lesions in Mice

We and others previously demonstrated that genetic deficiency of CD40L or treatment with neutralizing anti-CD40L antibodies slows both onset and progression of atherosclerotic lesions in LDLR\(^{-/-}\) mice.\(^{7,8}\) To explore the contribution of CD40 to atherogenesis, CD40\(^{-/-}\)/LDLR\(^{-/-}\) and LDLR\(^{-/-}\) mice consumed a high-cholesterol diet for 8 and 16 weeks. Total cholesterol and triglyceride levels did not differ statistically between the study groups (data not shown). Surprisingly, intimal lesion size of aortic arches was similar between CD40-deficient and control animals after 8 weeks (0.028±0.007 versus 0.037±0.007 mm\(^2\), \(P=\text{NS}\)) (Figure 1A) and 16 weeks of consumption of the high-cholesterol diet (0.254±0.032 versus 0.282±0.036 mm\(^2\), \(P=\text{NS}\)) (Figure 1A). Analysis of lesions in the aortic roots (Figure 1B) and thoracoabdominal aortas (Figure 1C) yielded similar results, which demonstrate that CD40 deficiency does not attenuate atherogenesis in these mice.

In agreement with our findings, the lesions of LDLR\(^{-/-}\) and CD40\(^{-/-}\)/LDLR\(^{-/-}\) mice did not differ in lipid and macrophage content after 8 weeks of high-cholesterol diet (30.1±6.3% versus 26.2±4.3% Oil-red-O–positive intimal area and 15.5±5.9 versus 19.6±3.4 Mac-3–positive intimal area, Figure 1D). After 16 weeks, CD40/LDLR compound deficient mice displayed even higher lipid- and macrophage-positive areas (37.4±4.9% versus 24±1.9% Oil-red-O–positive intimal area, \(P=0.023\); 45.5±7.8 versus 28±3.5 Mac-3–positive intimal area, \(P=0.036\)) (Figure 1D), which contrasted with our previous findings in CD40L-deficient and anti-CD40L antibody-treated mice.\(^{7,8}\) The differences in Oil-red-O content between abdominal aortas and aortic arches may in part reflect different stages in plaque development potentially caused by distinct flow and shear stress profiles.\(^{32,33}\)

#### sCD40L Binds to Mac-1

Four different experimental approaches tested the hypothesis that CD40L interacts directly with Mac-1: (1) Flow cytometry evaluated the binding of a flag-tagged recombinant sCD40L to nonstimulated and pamethoxyamphetamine (PMA)-stimulated human monocytes in the presence or absence of anti–Mac-1 antibody. sCD40L bound to monocytes, in particular to PMA-stimulated monocytes. An anti–Mac-1 antibody inhibited this binding (Figure 2A). (2) The binding of 125I-labeled sCD40L to Chinese hamster ovary (CHO) cells that expressed either nonactivated (CHO-Wt-Mac-1) or activated Mac-1 (CHO-Del-Mac-1) further supported the notion of a direct interaction between sCD40L and Mac-1. Similar to the results obtained with flow cytometry in monocytes, the activated Mac-1 bound strongly sCD40L, whereas the nonactivated Mac-1 bound only weakly (Figure 2B). In both cases, blocking anti–Mac-1 monoclonal antibody strongly reduced sCD40L binding (Figure 2B). (3) A blocking anti–Mac-1 antibody inhibited binding of radiolabeled sCD40L on activated monocytes, whereas control antibodies such as anti–P-selectin and anti-GP Ibβ/IIa antibodies did not inhibit sCD40L binding (Figure 2C). (4) Finally, direct binding of sCD40L was investigated with sCD40L as bait to precipitate its binding partner. Interestingly, sCD40L, precipitated activated Mac-1 expressed on CHO cells, but not nonactivated Mac-1 (Figure 2D). This observation agrees with the preferred binding of sCD40L to activated Mac-1 (Figure 2A through 2C). Overall, several lines of evidence support the concept of direct binding of CD40L to the integrin receptor Mac-1.

#### CD40L Mediates Cell Adhesion by Interaction With Mac-1

Static adhesion assays tested the functional relevance of the interaction between CD40L and Mac-1 for monocyte adhesion (n=6 each). Plates coated with sCD40L showed significantly greater monocyte adhesion than those coated only with agarose (106±3% versus 10±2%, \(P<0.05\)) (Figure 3A). Stimulation with PMA further enhanced CD40L-mediated adhesion (to 134±16, \(P<0.05\)) (Figure 3A). Preincubation with anti–Mac-1 antibody (100 µg/mL) limited both stimulated and nonstimulated leukocyte adhesion to sCD40L-coated plates (to 28±7 and 5±2%, \(P<0.008\), respectively) (Figure 3A), which demonstrated that CD40L–Mac-1 interaction mediates leukocyte adhesion.
To substantiate that Mac-1 can mediate cell adhesion on CD40L, we used the CHO cell lines that provided clearly defined states of Mac-1 affinity as well as control cells without any Mac-1 expression. Indeed, incubation of sCD40L-coated plates with CHO cells, CHO cells transfected with wild-type Mac-1 (CHO-Wt-Mac-1), and CHO cells transfected with permanently activated Mac-1 [GFFKR-deletion (Del-Mac-1)]\textsuperscript{34,35} affirmed the finding obtained with

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**Figure 1.** Generalized CD40-deficiency does not reduce atherosclerosis in mice. Early and advanced lesions of CD40\textsuperscript{-/-}/LDLR\textsuperscript{-/-} and LDLR\textsuperscript{-/-} mice are of similar size. CD40\textsuperscript{-/-}/LDLR\textsuperscript{-/-} and LDLR\textsuperscript{-/-} mice consumed a high-cholesterol diet for 8 and 16 weeks (n=12 animals per group), and underwent analysis of intimal lesion size in the aortic arch (A) and root (B). Pooled data±SEM are shown on the left; images of representative sections stained for lipid deposition (Oil-red-O) are displayed on the right. C, Lipid deposition in thoracoabdominal aortas from CD40\textsuperscript{-/-}/LDLR\textsuperscript{-/-} mice is similar to LDLR\textsuperscript{-/-} mice. The thoracoabdominal aortas from mice treated as described above were stained with Oil-red-O. The area positive for Oil-red-O in relation to total area is displayed as mean±SEM on the left and representative images of aortas are shown on the right. D, Advanced lesions of CD40\textsuperscript{-/-}/LDLR\textsuperscript{-/-} mice contain more lipids and macrophages compared with LDLR\textsuperscript{-/-} mice. Sections of the aortic arches of mice treated as described above were analyzed for lipid- and macrophage-specific staining. Oil-red-O– and Mac-3–positive staining in relation to intimal lesion size is displayed as mean±SEM. § P<0.05.
monocytes. CHO cells expressing the nonactivated Mac-1 (CHO-Wt-Mac-1), and even more so those that expressed activated Mac-1 (CHO-Del-Mac-1), displayed significantly enhanced adhesion compared with respective control CHO cells, an effect that again was reversed by an anti–Mac-1 antibody (Figure 3B). PMA stimulation in these recombinant cells did not increase the affinity of the Mac-1 receptors, but increased adhesion strength in general via stimulation of cytoskeletal rearrangements. Nevertheless, an anti–Mac-1 antibody specifically inhibited adhesion (data not shown). To validate that our observations in human cells also apply to mice, we isolated monocytes from a pool of 16 wild-type mice, and allowed them to interact with sCD40L-coated plates in the presence and absence of anti-Mac-1 antibody. Similar to our previous results, interaction of murine monocytes with sCD40L proved to be dependent on Mac-1 (Data Supplement Figure I).

**CD40L–Mac-1 Interaction Enhances Monocyte Migration In Vitro**

To test the hypothesis that CD40L not only binds to Mac-1 on monocytes, which results in the enhanced monocyte adhesion demonstrated above, but also promotes monocyte locomotion, we performed migration assays in a modified Boyden chamber. Soluble CD40L significantly enhanced monocyte migration compared with controls (28% versus 9%, P<0.05) (Figure 3C). Mac-1 mediates this effect, because pretreatment of monocytes with anti–Mac-1 antibody inhibited migration completely (to 2%, P<0.05) (Figure 3C).

**CD40L–Mac-1 Interaction Enhances Secretion of Myeloperoxidase by Monocytes**

To test the hypothesis that CD40L also promotes the release of prooxidant mediators from monocytes in a Mac-1–depen-
dent fashion, we assayed supernatants from static adhesion assays for myeloperoxidase (MPO) protein. Supernatants from plates coated with sCD40L contained significantly more MPO than those coated only with agarose (66±12% versus 25±3%, P<0.05; data not shown). Stimulation of monocytes with PMA further enhanced MPO secretion on sCD40L-coated plates (to 136±11, P<0.05) (Figure 3D), and anti–Mac-1 (100 ng/mL) antibody significantly inhibited both nonstimulated and stimulated secretion of MPO (to 30±5% and 46±9%, P<0.008, respectively) (Figure 3D).

**CD40L–Mac-1 Interactions Persist Under Flow Conditions**

We further tested whether CD40L–Mac-1 interactions persist under flow conditions relevant to those in human vessels. Under venous flow conditions, significantly more monocytes adhered on sCD40L-coated plates both without stimulation and in particular after PMA stimulation with a maximum after 5 minutes. Monocyte adhesion attenuated but persisted under simulated arterial flow conditions (Figure 4A and 4B). Again, pretreatment of monocytes with anti–Mac-1 antibody abrogated monocyte adhesion in both cases (Figure 4A and 4B), which corroborated the concept of direct CD40L–Mac-1 interaction that mediates leukocyte adhesion. The effects also persisted when arterial flow was applied from the beginning on, though overall adhesion fell markedly (data not shown).

**CD40L Induces Leukocyte Migration In Vivo via Interaction With Mac-1**

To test whether our in vitro observations extend to in vivo inflammation, we induced sterile peritonitis chemically in mice (n=3 per group). After intraperitoneal injection (4 hours) of 4% thioglycolate, peritoneal leukocyte counts were significantly lower in CD40L+/−/LDLR−/− mice (4.7±1.8×10⁶) than either LDLR−/− mice (9.0±0.7×10⁶, P<0.05) or CD40−/−/LDLR−/− mice (10.6±1.7×10⁶, P<0.05) (Figure 5A). Conversely, injection of anti–Mac-1 antibody (100 μg/mouse) 30 minutes before thioglycolate administration significantly attenuated macrophage infiltration in LDLR−/− mice (3.3±0.6×10⁶, P<0.05) and CD40−/−/LDLR−/− mice (3.4±0.6×10⁶, P<0.05) but not in CD40+/−/LDLR−/− mice (5.4±1.9×10⁶, P=NS) (Figure 5A). These data affirm that in contrast to CD40L–CD40 interactions, CD40L–Mac-1 participates pivotally in leukocyte recruitment in vivo. Additional experiments that involve injection of PBS or sCD40L (75 μg/mouse) intraperitoneally also demonstrated increased monocyte accumulation in the peritoneal fluid (5.1±0.9×10⁶ versus 3.0±0.3×10⁶, P=0.08) (Figure 5B), an effect reversed by pretreatment with anti–Mac-1 (100 μg/mouse) antibody (to 1.4±0.3×10⁶, P=0.02) (Figure 5B). Intraperitoneal injection of mock-transfected fibroblasts (WT-fibroblasts) or fibroblasts that overexpressed CD40L (CD40L-fibroblasts) for 4 and 72 hours further corroborated these findings (Figure 5C and 5D).
Inhibition of Mac-1 Attenuates Atherogenesis in Mice and Reduces Lesional Macrophage Accumulation

Because mechanisms that trigger peritoneal inflammation may be distinct from inflammatory processes that lead to atherogenesis, we tested whether Mac-1 modulates atherogenesis in vivo. Therefore LDLR<sup>−/−</sup> mice consumed a high-cholesterol diet for 10 weeks and received either 75 μg of anti-mouse Mac-1 antibody or carrier alone as control 3 times per week. Indeed, mice treated with anti–Mac-1 antibody developed smaller atherosclerotic lesions than respective controls (Figure 6A), which demonstrates a functional role of Mac-1 in atherogenesis. Immunohistochemical analysis of lesion composition demonstrated similar lipid content, but reduced macrophage accumulation in lesions of anti–Mac-1 treated animals, which suggests that Mac-1 promotes atherogenesis in vivo by increasing lesional macrophage invasion (Figure 6B and 6C).

Discussion

The present study made the surprising finding that CD40 does not contribute to CD40L-induced atherogenesis. Follow-up of this unexpected result led to the novel observation that CD40L interacts with the integrin Mac-1 and that these interactions can elicit monocyte adhesion and migration as well as MPO release in vitro and in vivo. These data uncover a novel mechanism of inflammatory signaling with implications for future treatment strategies that target CD40L.

Robust evidence implicates CD40L in the pathogenesis of atherosclerosis and its complications. Cells found in atheromata not only express both ligand and receptor, but the stimulation of endothelial cells, macrophages, and smooth muscle cells with CD40L triggers a broad proinflammatory response. In mice, genetic deficiency of CD40L and treatment with neutralizing antibodies that target CD40L decrease atherosclerotic lesion size, reduce preexisting disease, and also yield more fibrous plaques, a finding of potential clinical relevance. In contrast to expectations, our data suggest that CD40 does not mediate the proatherogenic functions of CD40L. Indeed, LDLR<sup>−/−</sup> mice that lack CD40 developed atherosclerotic lesions similar in size to those of corresponding control animals, irrespective of vascular location (arch, root, or abdominal aorta).

Macrophage invasion into the intima represents a key step in atherogenesis. We previously demonstrated that CD40L-deficiency reduces lesion size and macrophage content of mouse atheromata. In contrast, the present study demonstrates similar lesion size of and even enhanced macrophage content in lesions from CD40<sup>−/−</sup>/LDLR<sup>−/−</sup> mice, which suggests that CD40L-induced monocyte accumulation in plaques does not require CD40. The increased Oil-red-O and macrophage content in lesions from LDLR<sup>−/−</sup> mice that lack CD40 may even reflect a counterregulatory mechanism.
In light of these data and previous reports that describe interactions of CD40L with integrin family members, and in view the crucial role of Mac-1 in monocyte recruitment, we hypothesized that CD40L, soluble or membrane-bound, mediates some of its proatherogenic functions via interaction with the integrin Mac-1 on inflammatory cells.

Several lines of evidence establish a pivotal role for Mac-1 in monocyte adhesion to the endothelium, one of the initial steps of atherosclerotic lesion development. In contrast to other integrins such as LFA-1, recent work implicated Mac-1, particularly in the later, firmer adhesion under flow conditions. Furthermore, Mac-1 participates importantly in the transendothelial migration of monocytes that initiate a cascade of events that ultimately lead to plaque inflammation, growth, and destabilization. Rogers et al reported decreased intimal thickening after arterial balloon injury in rabbits after administration of Mac-1 antibodies. Simon et al extended these results to Mac-1-deficient mice, and thus demonstrated the in vivo relevance of Mac-1 in the arterial response to injury. Interestingly, Mac-1 binds to several other ligands apart from intracellular cell adhesion molecule-1, such as fibrinogen, lipoprotein (a), Thy-1 (CD90), receptor of advanced glycation end products, and vitronectin. Notably, several of these ligands associate with leukocyte adhesion/transmigration as well as atherogenesis.

Our data illustrate that CD40L interacts with Mac-1 present on monocytes, and that CD40L functionally enhances monocyte adhesion and migration in vitro in a Mac-1-dependent fashion. Furthermore, we demonstrate functional involvement of Mac-1 in atherogenesis in vivo, because Mac-1 inhibition in LDLR mice slowed lesion development and lesional macrophage accumulation. Growing evidence supports the concept that clustering of β2 integrins not only mediates cellular adhesion and migration, but also induces outside-in signaling, which results in altered gene expression. Thus, Mac-1 may participate in the expression of several proinflammatory cytokines including MIP-2,
interleukin-1β, interleukin-8, the procoagulant tissue factor, and in the activation of the proinflammatory transcription factor nuclear factor κ-B, which likely mediates the expression of the other factors.50–52 Interestingly, CD40L stimulation also elicits mediators induced by Mac-1 clustering on monocytes, and mediates many proinflammatory functions by nuclear factor κ-B activation.28 We show here that interaction of Mac-1 with CD40L enhances MPO release from monocytes. Recent work attributes proatherogenic functions to MPO,53,54 which suggests that CD40L not only may attract inflammatory cells via Mac-1, but also induces the expression of a variety of proinflammatory and prooxidant functions that promote atherogenesis.

Our present findings do not counter the multitude of reports that demonstrate CD40L-dependent immunologic functions of CD40L.55,56 CD40L may very well exert its immunologic effects as costimulatory molecule in the T-cell-dependent humoral immune response via its classic receptor, yet its proinflammatory properties that promote chronic inflammatory diseases such as atherosclerosis may depend on interactions with other partners such as Mac-1. Indeed, a previous study suggested the existence of CD40-independent CD40L-induced functions. Mehlihp et al observed that bronchial hyperresponsiveness induced by Aspergillus inhalation requires CD40L but not CD40. They proposed a receptor-independent direct effect of CD40L on T lymphocytes or an interaction with an unknown other receptor on other leukocytes (eg, basophils).57 Conversely, others have postulated the existence of alternative ligands for CD40.58 Furthermore, other data show an interaction of CD40L with members of the integrin family. André et al demonstrated that CD40L stabilizes arterial thrombi by a αvβ3-integrin-dependent mechanism, a function not modulated by the presence or absence of CD40.14 Later, Prasad et al showed β3 integrin tyrosine phosphorylation induced by CD40L as an underlying mechanism.15 Although this interaction with αvβ3 may explain the prothrombotic functions of CD40L, the interactions with Mac-1 reported here may contribute more to the proinflammatory functions of CD40L on the plaque per se.

Our data from the present study do not establish that CD40L–Mac-1 interactions represent the exclusive mechanism of CD40L-induced inflammation. Mac-1 is rather specific for cells of the myeloid lineage, and CD40L exerts proinflammatory effects in a variety of cell types such as vascular smooth muscle and endothelial cells.2,6 CD40 as well as other receptors on these cell types may mediate some of CD40L-induced proinflammatory functions. CD40L binding to GP IIb/IIIa on platelets may also participate in atherogenesis. Similarly, some of the CD40-dependent immunologic functions of CD40L may contribute to atherosclerosis and many other inflammatory diseases. Our data highlight the pivotal role of CD40L in inflammation and inflammatory diseases such as atherosclerosis by uncovering a new mechanism of CD40L-induced inflammation. The present observations have important implications for both the mechanisms of inflammatory diseases as well as their therapeutic modulation.

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**Disclosures**
None.
References


**CLINICAL PERSPECTIVE**

Atherosclerosis is recognized as a specific form of inflammatory disease of the arterial vessel wall. From the very beginning of atherogenesis to the final rupture of atherosclerotic plaques, leukocytes, in particular monocytes/macrophages, play an essential role. One of the molecules that mediate atherogenesis is CD40 ligand (CD40L; CD154), a transmembrane protein that was first described on T lymphocytes that participated in T-cell–dependent humoral immunity. Animal models established its role as a mediator of atherosclerosis. Epidemiological studies identified the shed-soluble extracellular part of CD40L as a potent risk marker for cardiovascular events in humans. CD40 is the classic receptor for CD40L. The interaction of CD40L with CD40 on B cells directs immune responses. Cell types dominant in atherosclerosis such as T cells, endothelial cells, smooth muscle cells, platelets, and monocytes/macrophages also express CD40. Thus, the general yet unproven expectation is that CD40 plays a pivotal role in atherosclerosis. In the present study, however, we demonstrate in mice that CD40L mediates atherogenesis independently of CD40. By different experimental approaches, we identify the integrin Mac-1 as an alternative receptor for CD40L that mediates CD40L's proatherogenic properties. This novel mechanism for CD40L-induced inflammation may lead to novel approaches in antiinflammatory and antiatherosclerotic therapy.
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