The multi-functionality of CD40L and its receptor CD40 in atherosclerosis

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Summary
Disrupting the CD40-CD40L co-stimulatory pathway reduces atherosclerosis and induces a stable atherosclerotic plaque phenotype that is low in inflammation and high in fibrosis. Therefore, inhibition of the CD40-CD40L pathway is an attractive therapeutic target to reduce clinical complications of atherosclerosis.

The CD40-CD40L dyad is known to interact with other co-stimulatory molecules, to activate antigen-presenting cells (APC) and to contribute to T-cell priming and B-cell isotype switching. Besides their presence on T-cells and APCs, CD40 and CD40L are also present on macrophages, endothelial cells and vascular smooth muscle cells in the plaque, where they can exert pro-atherogenic functions. Moreover, recent progress indicates the involvement of neutrophil CD40, platelet CD40L and dendritic cell CD40 in atherogenesis. Since systemic CD40-CD40L modulation compromises host defense, more targeted interventions are needed to develop superior treatment strategies for atherosclerosis. We believe that by unravelling the cell-cell CD40-CD40L interactions, inhibition of cell-type specific (signalling components of) CD40(L) that do not compromise the patient’s immune system, will become possible. In this review, we highlight the cell-type specific multi-functionality of CD40-CD40L signalling in atherosclerosis.

Keywords
Atherosclerosis, immunity, inflammation, co-stimulation, TNF receptor-associated factor

Introduction
In the late 1990s it became clear that atherosclerosis is a chronic inflammatory disorder of the arterial wall (1–3). Today we know that the process of atherosclerosis is a complex interplay between modified lipids, different cells of the immune system, endothelial cells and smooth muscle cells. Even more, the understanding of the pathophysiology of atherosclerosis entered a new era. Elegant new studies describe the diversity of cell types involved in atherogenesis (4). Besides the well known cell-types like monocytes, T cells and B cells, different monocyte subsets (e.g. Ly-6Chi, Ly-6Clo) (5–8) and T-cell subsets (e.g. CD4+ T-helper cells [Th1, Th2, Th17], CD8+ cytotoxic T cells, regulatory T cells) (9) have been described to be present in plaques and to be causally implicated in the disease process. Furthermore, unexpected roles for neutrophils (10, 11), mast cells (12, 13), dendritic cells (14) and platelets (15, 16) in atherosclerosis were recently discovered.

A receptor-ligand pair that has been shown to play an important role in atherosclerosis is the CD40-CD40L dyad. CD40 is a member of the tumour necrosis factor receptor (TNF-R) superfamily (17) that is activated by CD40 ligand (CD40L, also known as CD154), a 39 kD transmembrane glycoprotein and member of the tumor necrosis factor α (TNF-α) family. CD40L can also be shedded into a soluble (s)CD40L form. sCD40L is a truncated form (18 kDa) of the CD40L protein (18). sCD40L retains the ability to bind the CD40 receptor as a trimeric ligand and is believed to be biologically active. However, its repertoire of functional activities and potential differences between CD40L and sCD40L remain to be further elucidated.

CD40-CD40L interactions are originally known as co-stimulatory molecules indispensable for the function of antigen-presenting cells and activated CD4+ T cells. Nevertheless, CD40 and CD40L are both expressed on the vast majority of immune cells (lymphocytes, monocytes, dendritic cells, neutrophils and mast cells) and non-immune cells (e.g. endothelial cells and vascular
CD40-CD40L signalling induces cell-proliferation, -differentiation and -activation, which is characterised by the release of a wide range of cytokines (e.g. interleukin [IL]-1β, IL-2, TNF-α, interferon [IFN]-γ) and growth factors (VEGF) that exert many pro- and anti-inflammatory effects (20, 21). Furthermore, CD40-CD40L signalling results in the expression of chemokines (e.g. MCP-1, RANTES) and endothelial adhesion molecules (e.g. ICAM-1, VCAM-1, P-selectin) that stimulate trafficking of leukocytes and regulate the migration of different cell types (22, 23).

Both CD40 and CD40L are present in human atherosclerotic lesions. Macrophages, smooth muscle cells, endothelial cells and T cells express CD40 and CD40L in fatty streaks and the expression increases in advanced human atherosclerotic lesions (24–26). CD40-CD40L interactions mediate several of the processes that set the stage for plaque rupture, like degradation of the extracellular matrix and formation of the necrotic core (19). Moreover, we, and others, showed that mice deficient in CD40L have less atherosclerosis and develop plaques that are low in inflammation and high in fibrosis, generally referred to as a stable plaque phenotype (27–30). This review will focus on the potential atherogenic effects of CD40-CD40L signalling in the individual cell types relevant to atherosclerosis and their impact on the overall effect of CD40 signalling on plaque formation and stability.

**CD40-CD40L inhibition in atherosclerosis**

Multiple intervention studies have revealed the pro-atherogenic effects of CD40-CD40L signalling. In 1998, Mach et al. showed that hyperlipidemic LDLR-/- mice treated with an anti-CD40L antibody significantly reduced the size and lipid content of aortic atherosclerotic lesions (30). In 1999, we showed that CD40L-/-Apoe-/- mice exhibited a 5.5 fold decrease in plaque area (27). Moreover, CD40L-/-Apoe-/- animals contained pronounced collagen-rich, lipid-poor advanced plaques with a decreased macrophage and T-cell content. In a follow-up study, we used an anti-CD40L antibody (MR-1) to block CD40L signalling (29). Anti-CD40L was administered in ApoE-/- mice on normal chow diet, either at the onset of atherosclerosis or when established atherosclerotic lesions were already present. In both the early and delayed treatment groups, anti-CD40L antibody treatment did not result in a decrease in plaque area but resulted in the development of a lipid-poor, collagen-rich stable plaque phenotype. Schonbeck et al. showed similar results with an anti-CD40L antibody (M158, Immunex) treatment in LDLR-/- mice that were on high-fat diet (30–32).

These findings provided novel insights in the inflammatory pathway of atherosclerosis and identified CD40-CD40L interactions as a promising therapeutic target to improve plaque stability, even when established atherosclerotic lesions are present. Thus, inhibition of CD40-CD40L interactions is expected to prevent clinical complications of atherosclerosis, thereby reducing (atherosclerosis)-related morbidity and mortality. It has been suggested that, long-term inhibition of the CD40-CD40L axis might severely compromise the immune system of the patient, thereby hampering the therapeutic potential of this target. However, in a phase I trial, using a monoclonal anti-CD40L antibody (IDEC-131) for patients suffering from systemic lupus erythematosus (SLE) no side effects were observed (33). Patients received a single intravenous injection. However, no further development for this indication has been reported (34). In addition, Schuler et al. tested a humanised CD40L monoclonal antibody (ABT793) in renal allografttransplantsations (35). ABT793 treatment indeed effectively prevented graft rejection in cynomolgus monkeys. Since treatment was accompanied by thromboembolic events, all clinical trials were put on hold. In these studies, only a single or a couple of doses were administered. In order to contribute to plaque stabilisation, long-term treatment will be necessary, which may even lead to more severe and frequent adverse side effects. However, as a potential alternative to CD40L therapy targeting of CD40, the receptor for CD40L would be feasible. In allotransplantation research, two promising anti-CD40 antibodies were developed: Ch220 in 2002 and 4D11 in 2007 (36, 37). Both antibodies appear to be an encouraging agent for anti-rejection treatment in clinical organ transplantation. Satisfying prolonged allograft survivals in monkeys were obtained without thromboembolic complications. Both antibodies open a new prospective for inhibition of the CD40-CD40L system in atherosclerosis.

Given the complex, cell specific mode of action of CD40 signalling, we believe it is necessary to study cell-type specific CD40-CD40L signalling. This will enable us to identify the cell-cell CD40-CD40L interactions that are responsible for the destabilisation of the atherosclerotic lesion and thus to target CD40-CD40L signalling in a cell-specific manner with minimal side effects. In addition, it will be essential to elucidate the cell-type specific CD40-CD40L signalling cascade in order to find downstream targetable molecules that will allow targeted blockage of CD40 signalling in an atherosclerosis-specific way.

**The CD40-CD40L system**

CD40 lacks intrinsic signalling activity and needs to recruit adaptor molecules upon activation. Ligation of CD40 to CD40L results in trimerisation of CD40 and subsequent association of adaptor proteins (TNF receptor-associated factors, or TRAFs) to its cytoplasmic domain. The TRAF protein family is composed of six members (TRAF 1, 2, 3, 4, 5 and 6). CD40 can bind five of the six TRAF members (TRAF 1, 2, 3, 5 and 6), depending on cell type and function. CD40 has three TRAF binding domains, one for TRAF6 and one for TRAF2&3 and, indirectly, TRAF5. Recently, Lu et al. identified a new functional domain within the cytoplasmic tail of CD40 that contains an alternative TRAF2 binding-site (38). These binding sites can initiate different downstream signalling pathways resulting in a broad range of cell-type specific actions of CD40-CD40L. For example, in B lymphocytes, CD40-TRAF6 interactions are required for CD40-mediated IgM production, isotype switching and B cell-induced IL-6 secretion, whereas the TRAF2/3 binding site is required for CD40-mediated upregulation of the co-stimulatory molecules B7–1 and B7–2 (39, 40). Signalling results in the activation and nuclear
translocation of different transcription factors, e.g. nuclear factor kappa B (NF-xB), nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) leading to the production of pro-inflammatory and pro-atherogenic cytokines/chemokines, growth factors (e.g. VEGF), matrix metalloproteinases (MMPs), and adhesion molecules, depending on the cell-type and CD40-TRAF binding site involved (19).

Only scattered information exists on CD40-TRAF signalling that is induced by sCD40L (41). When CD40L binds to CD40 on endothelial cells, CD40 is internalised into the membrane associated lipid raft fraction, where binding of TRAF1/2/3/5 and/or TRAF6 occurs, followed by activation of NF-xB and subsequent induction of inflammation. However, when sCD40L binds to CD40, rapid endocytosis of the CD40 receptor is induced independently of TRAF2/3/5/6 binding. Upon binding of sCD40L to CD40, CD40 translocates to the early, rab5+ endosome, activates NF-xB but fails to induce expression of pro-inflammatory cytokines and adhesion molecules. Since only scattered, but important information on this subject is available, further characterisation of the signalling network of the different forms of CD40L, as well as of the different CD40-expressing cell-types is needed.

The discovery of this complex signalling pathway heralds a new area in the development of therapeutic agents with limited side effects. Different cell types have their own specific TRAF-mediated CD40-CD40L signalling pathway. Therefore, we need to find the CD40-TRAF interactions important in atherosclerosis as well as the cell-type specific CD40-TRAF signalling.

In a recent study we induced neointima formation in mice that express a human/mouse chimeric CD40 transgene (human CD40 extracellular domain; mouse CD40 transmembrane and cytoplasmic domain) under a major histocompatibility complex (MHC) II promoter and contains mutations at the TRAF2,3&5 (CD40-T2/3/5), TRAF6 (CD40-T6), or both the TRAF2/3/5 and TRAF6 (CD40-T2/3/5&6) binding site. Using these unique mouse models we were able to determine the leukocyte specific CD40L signalling in relationship with neointima formation (42). In this study we identified TRAF6 as the key player in CD40L signalling in leukocytes in neointima formation. Moreover, CD40-TRAF6 signalling was required for inflammatory cell infiltration and collagen turnover in the neointima (42).

Even though we have identified the key factors in CD40 signalling, the exact mechanisms still need to be unravelled. Elucidating the exact mechanisms will open opportunities to develop more specialized treatment with fewer side effects.

Cell type-specific CD40-CD40L signalling

It has become evident that CD40 and/or CD40L are expressed on the majority of cell types involved in atherosclerosis (Table 1). In these paragraphs we will elaborate on the (pro-atherogenic) consequences of CD40-CD40L signalling of the distinct cell-types that have a role in plaque progression and destabilisation (Table 2).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CD40</th>
<th>CD40L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>resting</td>
<td>activated</td>
</tr>
<tr>
<td>CD4+ T-cells</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>B-cells</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Platelets</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>SMCs</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1: Cell types expressing CD40 and CD40L.

- , no expression; + weak expression; ++, average expression; ++++, strong expression. SMC, smooth muscle cells.
In T-cell receptor (TCR)-activated cells, calcium was sufficient to induce membrane CD40L expression, but insufficient for the production of sCD40L. Furthermore, the study showed that CD40L shedding is protein kinase C (PKC) dependent, involving the activity of a Zn2⁺-dependent matrix metalloproteinase. They further suggest that cleavage by the ADAM-10 protease, which belongs to a superfamily of Zn2⁺-dependent metalloproteinases, may represent an important mechanism for sCD40L generation. However, we have to state that the biological effects of T cell-derived sCD40L remain to be determined and we have to take into account that sCD40L is mainly shed from platelets and not from T-cells.

Interestingly, T-cells can also express CD40 (49). However, the mechanisms of T-cell CD40 function have not been clearly defined. In 2002, Bourgeois et al. demonstrated that in addition to CD40L, both activated CD4+ and CD8+ T-cells also expressed its receptor CD40 (50). In the absence of CD40, CD8+ T-cells were unable to differentiate into memory cells or receive CD4 help. These results suggest that, like B cells, CD8+ T cells receive CD4 help directly through CD40 and that this interaction is fundamental for CD8+ memory T-cell generation.

We, and others, investigated the effects of leukocyte CD40L on atherosclerosis by transplanting CD40L-/- bone marrow into LDLR-/- recipients (46, 51). Surprisingly, no effect on plaque progression or phenotype could be found. These data reveal that bone marrow-derived CD40L-expressing cells alone are not responsible for the stable plaque phenotype observed in CD40L-/-/ApoE-/- mice. However, bone-marrow transplantation cannot address the function of individual haematopoietic lineages and more precise studies have to be performed to elucidate the specific role of T-cell CD40L in atherosclerosis.

### Table 2: Cell type-specific CD40-CD40L signalling

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell type</th>
<th>Effect</th>
<th>Induced by</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>APC</td>
<td>Up-regulation of co-stimulatory activity (ICAM-1, VCAM-1, E-selectin, LFA-3, B7-1, B7-2, MHCI-II, CD40)</td>
<td>T-cell CD40L</td>
<td>9, 43, 44</td>
</tr>
<tr>
<td></td>
<td>B cell</td>
<td>Isotype switching</td>
<td>T-cell CD40L</td>
<td>52,53</td>
</tr>
<tr>
<td></td>
<td>Monocyte</td>
<td>IL-12, IL-1β secretion</td>
<td>T-cell CD40L</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>secretion of cytokines (e.g. IL-12, TNF-α, IL-1β, IL-6, IL-8)</td>
<td>T-cell CD40L</td>
<td>43,62, 63</td>
</tr>
<tr>
<td></td>
<td>Platelet</td>
<td>Platelet activation</td>
<td>T-cell CD40L sCD40L</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Neutrophil</td>
<td>ROS Enhanced MAC-1 expression</td>
<td>Plt CD40L sCD40L</td>
<td>83,84</td>
</tr>
<tr>
<td></td>
<td>DC</td>
<td>IL-12 secretion</td>
<td>T-cell CD40L</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Endothelial cell</td>
<td>Upregulation of adhesion receptors (E-selectin, VCAM-1, ICAM-1)</td>
<td>T-cell CD40L Plt CD40L sCD40L</td>
<td>90,91,92,93, 94, 95</td>
</tr>
<tr>
<td></td>
<td>αSMC</td>
<td>Chemokines (MCP-1, IL-8) IL-1β, tissue factor</td>
<td>98,99, 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α1β3</td>
<td>Platelet Stabilisation of arterial thrombi</td>
<td>sCD40L</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Mac-1</td>
<td>Monocyte Mediates monocyte adhesion</td>
<td>sCD40L</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>α5β1</td>
<td>Monocyte Mediates monocyte adhesion</td>
<td>sCD40L</td>
<td>(103)</td>
</tr>
</tbody>
</table>
CD40-CD40L interactions play a crucial role in humoral, adaptive, immune responses. T-helper cells that are activated by antigens and co-stimulatory molecules (B7-CD28), express CD40L that binds to CD40 on B cells where it is constitutively expressed. This interaction, together with cytokines produced by the T-helper cell, will stimulate B-cell proliferation and differentiation into plasma cells. Consequently, B cells will undergo Ig-isotype switching. In the absence of signals from T-helper cells (CD40L, cytokines), B cells will only produce IgM (Fig. 1). However, in the presence of signals from T-helper cell CD40L, B cells will undergo isotype switching to other Igs (IgG subclasses, IgE and IgA) (52, 53). In humans, mutations in the CD40L gene are associated with the X-linked hyper IgM syndrome, characterised by low or absent levels of IgG, IgE and IgA in serum, but normal or elevated serum-levels of IgM (54).

B cells play a significant role in atherosclerosis. They are not commonly found in lesions, but are present in the adjacent adventitia of established lesions and are thought to have an atheroprotective function. Caligiuri et al. showed that splenectomy dramatically aggravated atherosclerosis in hypercholesterolemic ApoE/-/- mice and decreased titers of IgM anti-OxLDL antibodies, effects that could be rescued by infusion of B cells from aged apoeE/-/- mice (55). In addition, Binder et al. demonstrated that Th2 cells produce, during atherogenesis, large amounts of IL-5, which in turn stimulates B-1 cells to secrete T15/E06 (oxLDL specific) IgM antibodies (56). Despite the fact that B cells are important in atherosclerosis, the exact role of B cell-specific CD40 signalling in atherosclerosis remains to be determined.

Monocytes/macrophages
A myriad of monocyte and macrophage subsets are currently subject of numerous studies. However, their functional significance in atherosclerosis is still under debate and the exact role of CD40-CD40L signalling in monocyte and macrophage differentiation into the different subsets still needs to be elucidated. Therefore we will focus in this paragraph on general CD40-CD40L signalling in monocytes and macrophages rather than the effect on the different subsets.

Several reports describe functional CD40-CD40L interactions between T cells and monocytes (57). CD40 is constitutively expressed on monocytes and activated T cells are able to activate monocytes through CD40-CD40L signalling (58). This interaction is bidirectional and activated monocytes are capable of activating other T cells. Ligation of CD40 on monocytes will induce IL-12 secretion, which induces CD40L expression on T cells (59) and IL1-β, a pro-inflammatory cytokine, abundantly expressed in the atherosclerotic lesion.

Most biological functions of CD40L have been attributed to direct interaction with CD40, the classical receptor for CD40L. However, alternative receptors have been described in recent years. Mehlhop et al. showed that the development of bronchial hyper-responsiveness could be prevented in CD40L/-/- mice but not in CD40/-/- mice, indicating for the first time that CD40L can bind other receptors then CD40 (60). More recently, Zirlik et al. showed that CD40L can also interact with Mac-1 present on monocytes, and that CD40L improves monocyte adhesion and migration as well as myeloperoxidase release in vitro in a Mac-1-dependent way (61).

CD40L on T cells also binds to CD40 on macrophages, which in turn become activated. Activated macrophages are able to synthesize and secrete matrix metalloproteinases (e.g. MMP-1, MMP-2, MMP-3, MMP9) (62), which are known to degrade extra-cellular collagen matrix, thereby weakening the cap of the plaque (63). Even more, ligation of CD40 on macrophages evokes the release of pro-inflammatory cytokines (IL-12, TNF-α, IL-1β, IL-6, and IL-8). The CD40L-CD40 system also affects thrombosis as it induces macrophages to secrete tissue factor (62).

Platelets
Several lines of evidence suggest that platelets not only promote thrombus formation, but also propagate inflammatory processes. Platelets, upon activation, can induce inflammation directly by autocrine and paracrine mechanisms. Activated platelets release their α-granules, which contain, besides clotting proteins, a plethora of cytokines, chemokines and growth factors.

Detection of activated platelets, as defined by P-selectin surface expression, in peripheral blood of patients with unstable angina pectoris was first reported by Fitzgerald et al. (64). Multiple studies were recently published that presented insights into the inflammatory function of activated platelets in the process of atherosclerosis (15, 65, 66). Huo et al. showed that circulating activated platelets promote monocyte recruitment to atherosclerotic arteries and accelerate atherosclerosis in ApoE-deficient mice (16). The increase in lesion area was halted when the activated platelets were P-selectin negative. Moreover, platelets that transiently interact with the atherosclerotic endothelium are able to deliver and deposit the chemokines RANTES (CCL5) and PF4 (CXCL4). Both RANTES and PF4 deposition by platelets promotes atherosclerosis by triggering monocyte arrest on the endothelium of atherosclerotic lesions (67, 68).
Besides the direct effect, platelets induce inflammation by indirect interactions with other cell types, especially endothelial cells and monocytes. Henn et al. proved that platelets, through CD40-CD40L interactions, are capable to initiate various inflammatory responses on endothelial cells (Fig. 2) such as the expression of inflammatory adhesion receptors (e.g. E-selectin, vascular adhesion molecule-1 [VCAM-1], intercellular adhesion molecule-1 [ICAM-1]) the production of chemokines (e.g. monocyte chemoattractant protein-1 [MCP-1], interleukin-6 and interleukin-8) and the production of matrix metalloproteinase 9 (MMP9) (66, 69). Dole et al. showed that activated platelets promote the secretion of Weibel-Palade bodies and leukocyte rolling, which is mediated by platelet P-selectin and not by CD40L (70).

Another important feature of activated platelets is the capability of binding monocytes to form platelet-monocyte aggregates (71). Platelets will hereby stimulate monocytes to differentiate into a more pro-adhesion and pro-migratory phenotype and promote monocyte recruitment to the lesion (72). Since more than 95% of circulating CD40L exists in platelets, we believe that platelet CD40L could play a decisive role in the pathogenesis of atherosclerosis (Fig. 3). CD40L is stored in the cytoplasm of resting platelets and rapidly presented at the surface after activation. CD40L on platelets can interact with CD40 that is present on leukocytes (preferentially monocytes), and form platelet-leukocyte-aggregates (PLA). In humans with profound atherosclerosis, elevated numbers of PLA are found in the blood, which result in more cytokine production (73).

Upon platelet activation, CD40L is cleaved from the platelet surface over a period of minutes to hours to generate a 18 kDa sCD40L molecule, which is similar to the T cell-released sCD40L. The release of sCD40L is much slower then α-granule release and therefore not a direct result of α-granule release. Clinical studies were carried out to evaluate the value of sCD40L levels as biomarker for cardiovascular risk. Elevation of sCD40L indicated an increased risk of cardiovascular events (74). sCD40L is known to be pro-thrombotic via stabilisation of arterial thrombi in a β3-integrin-dependent mechanism (75) and it has been reported that αIIbβ3 (GP IIb/IIIa) antagonists inhibit the release of sCD40L from activated platelets (76). In addition, Henn et al. reported that CD40 is constitutively expressed on platelets, and that the interaction between CD40 and CD40L is needed for the shedding of sCD40L (77). Unfortunately, it is not known if platelet derived sCD40L is cleaved in the same way as T cell-derived sCD40L. However, recently, in Crohn disease patients, a specific inhibitor of MMP-9 was able to significantly reduce platelet CD40L shedding, suggesting a Zn\(^{2+}\)-cleavage site (78). Interestingly, in endothelial cells, only membrane-bound CD40L is able to induce expression of pro-inflammatory cytokines and adhesion molecules, whereas sCD40L is not (41). However, Chen et al. showed in 2008 that sCD40L could induce endothelial dysfunction by significantly decreasing endothelial nitric oxide synthetase (eNOS) expression and nitric oxide (NO) production.

![Figure 2: Platelet-endothelium adhesion through CD40-CD40L induces the release of pro-inflammatory mediators and tissue factor and the expression of adhesion molecules.](image)

![Figure 3: Potential pro-atherogenic functions of CD40-CD40L signalling during atherogenesis.](image)
production (79). Although there is still some controversy on the function of sCD40L, we have to consider the potential of sCD40L to mediate inflammatory events within the vasculature (76).

Besides CD40L and sCD40L, platelets are also shown to express the receptor CD40. The role of this platelet CD40 is still under debate. However, Danese et al. showed that CD40L-positive T cells induced platelet activation through a contact-mediated, CD40-dependent pathway. This resulted in RANTES release, which bound to endothelial cells and mediated T-cell recruitment (80). Soluble CD40L induced the same events.

Finally, Crist et al. showed that CD40L is also abundantly expressed on the membrane of megakaryocytes and found that NFAT, a calcium-dependent transcriptional regulator associated with activated T cells, mediated both differentiation-dependent and inducible megakaryocyte-specific CD40L expression (81).

Neutrophils

The concept of neutrophils as strong mediators of atherosclerosis development is emerging. The presence of neutrophils and myeloperoxidase in murine lesions was described by van Leeuwen et al. (10). Even more, Soehnlein et al. described that neutrophils, through their secretory products, pave the way for inflammatory monocytes in the progress of atherogenesis (11). Zernecke et al. showed that the CXC ligand (CXCL)12/CXC receptor (CXCR4) chemokine-receptor axis controls the important contribution of neutrophils to atherogenesis in mice (82). These studies illustrate that as a cell type with a major role in mediating inflammatory responses, the neutrophil could be an important mediator in the development of atherosclerosis.

CD40 was reported to be present on neutrophils, although its precise role remains to be determined. Neutrophils are able to interact with platelets through CD40-CD40L interactions. These interactions appear to influence both cell-types. Vanichakarn et al. demonstrated that stimulated platelets activate neutrophils through the release of soluble CD40L (83). This sCD40L will stimulate neutrophils, via CD40, to release reactive oxygen species (ROS), which subsequently will stimulate more platelets. In another elegant study, by Li et al., incubation of mouse neutrophils with activated platelets led to enhanced MAC-1 expression on neutrophils (84). Anti-CD40L and anti-P-selectin antibodies inhibited this effect. The same study showed that elevated levels of sCD40L promoted platelet-leukocyte activation and recruitment, as well as neointima formation after arterial injury. These findings suggest a novel mechanism in the recruitment of leukocytes and the formation of platelet-leukocyte aggregates through CD40-CD40L signalling.

Dendritic cells

APC, especially dendritic cells (DCs), the central cell type of the immune system, immediately sense potential pathogens and provide the first link to an adaptive immune response by activating T cells. CD40-CD40L co-stimulatory signalling plays a crucial role in DC function. Immature DCs in the periphery take up antigens efficiently but express low levels of MHC and co-stimulatory molecules like CD40, resulting in a poor capacity to stimulate T cells. However, upon maturation, DCs significantly augment their ability to stimulate naïve T cells by surface exposure of antigen/MHC complexes and by upregulation of CD40 and other co-stimulatory (e.g. B7–1, B7–2) molecules. DCs consist of numerous distinctive subsets, with diverse immunological functions, unique markers and different tissue distributions. Several dendritic-cell-classifications have been described. The commonly used DC phenotypes are the CD11ch+ MHCII+ conventional DCs (cDCs), which are subdivided into migratory and lymphoid-tissue-resident DCs, and the type I interferon-producing plasmacytoid DCs (pDCs). It has been shown that DCs, upon constant CD40L stimulation, provide long-lasting IL12 responses, important for driving a potent Th1 response (85). It has also been revealed that TRAF6 is important for DC maturation, cytokine production, and T-cell stimulatory capacity of DCs in response to CD40 ligand, but also for the homeostasis of splenic DC subsets (86). In addition, Kuwajima et al. demonstrated recently that CD40 on pDCs could interact with CD40L on pDCs, resulting in IL-12 production by pDCs (87). This study demonstrates the importance and the difference of CD40-CD40L signalling between different subsets of DCs.

In atherosclerosis, the function and distribution of DC subsets still remains to be elucidated (4, 88). Together with the fact that naïve T cells require priming by DCs in secondary lymph nodes in order to enter peripheral tissues like atherosclerotic plaques, CD40-CD40L interactions between dendritic cells and T cells could be an interesting target to study in atherosclerosis.

Endothelial cells and vascular smooth muscle cells (vSMC)

CD40-CD40L interactions play a significant role in the activation of vascular endothelium (89). Endothelial cells, as well as vSMC express both CD40L and its receptor CD40. Enhanced adhesion of immuno-competent cells to the endothelium may be among the earliest pro-atherogenic functions mediated by the CD40-CD40L dyad. Ligation of CD40 on endothelial cells induces the expression of several cell adhesion molecules (VCAM-1, E-selectin, ICAM-1) and chemokines (IL-8, MCP-1 and MIP-1α) (90, 91). This will lead to the recruitment of monocytes and lymphocytes to the site of injury. IL-1, IL-6 and TNF-α also play a part in this process along with CD40L (92). Other elements involved are macrophage inflammatory protein-1 (MIP-1), RANTES and chemoattractant protein-1 (MCP-1). Soluble CD40L may also contribute to endothelial dysfunction by reducing NO through an increased generation of ROS and promote atherogenesis (93, 94). Bavendiek et al. showed that ligation of CD40 on endothelial cells induces tissue factor expression (95). On the subject of signalling, Zirlik recently showed that CD40L enhances endothelial expression of TRAF-1,-2,-3 and 6 but not TRAF-5 (96). Furthermore, TRAF-associated signalling induced by CD40L differs from activation pathways used by other pro-inflammatory cytokines like TNF-α or IL-1β.

In relation to CD40-CD40L interactions in vSMC, Hermann et al. showed that CD40L can activate mitogenic signalling and DNA synthesis in VSMC but does not contribute to proliferation or migration of vascular SMC (97). CD40 signalling in vascular SMC activates a Src family kinase-initiated pathway that results in the induction of MAPK activities like the chemokines MCP-1 and IL-8 (98). In addition, Schonbeck showed already in 1997...
that ligation of CD40 on vSMC activates IL-1β-converting enzyme (caspase-1) that promotes IL-1β production (99). In another publication by the same group, ligation of CD40 on vSMC was found to mediate the loss of interstitial collagen by the production of several metalloproteinases (MMP) (100). These MMPs weaken the plaque cap and promote plaque rupture (101). Finally, the CD40-CD40L signalling pathway also regulates tissue factor activity in vSMC (31).

Concluding remarks

Given the plaque-stabilising capacity of CD40L blockage, CD40-CD40L interactions have been appreciated as a promising therapeutic target to reduce clinical manifestations of atherosclerosis, and thus thereby morbidity and mortality. However, long-term systemic inhibition of total CD40(L) can result in immune-suppression.

This review highlighted the potential contribution of each of the different CD40(L) expressing cell types, as well as the most important circulating source of CD40L: platelets, to atherosclerosis. Important functions in the context of atherosclerosis have been established for CD40-CD40L signalling in monocytes, T cells and B cells. We believe that platelet CD40L and sCD40L could play a decisive and central role in the inflammatory process of atherosclerosis by orchestrating both the activation and recruitment of different leukocyte subsets to the vessel wall. In addition, neutrophils, constitutively expressing CD40, present an attractive new approach in understanding the mechanisms of atherosclerosis. However, further research is needed to determine the exact role of neutrophils and especially neutrophil CD40. It will be particularly important to extrapolate these findings to human disease, where the contribution of neutrophils is unclear. Finally, the understanding of CD40 in DC function in atherosclerosis is still under investigation (102). Different subsets of DCs with distinct functions have been described, but their specific role in atherosclerosis is yet to be determined (102). Therefore, we believe that dendritic cell CD40 in relation to atherosclerosis could be a promising target to study.

In order to develop a targeted, cell-type based, therapeutic agent with limited side effects the complex equilibrium between the CD40(L)-expressing cell types has to be further elucidated.

References