

# CD40 Ligand and Its Receptors in Atherothrombosis

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## 1. Introduction

Atherothrombosis is the main underlying determinant of cardiovascular diseases, which remain the leading cause of death in developed countries. Multiple lines of evidence now support the concept of atherothrombosis as a chronic inflammatory disease of the arterial wall.<sup>1, 2</sup> This process involves a complex interplay between modified lipids and cells of the immune and vascular system, which usually evolves into the formation of atherosclerotic lesions yielding a stable necrotic plaque. If left untreated, plaque rupture and thrombosis may ensue, leading to important clinical manifestations, such as acute coronary syndromes and sudden death.<sup>3</sup>

As the incorporation of modified low-density lipoproteins in the arterial wall represents an important step in the onset of atherothrombosis, the subsequent recruitment and activation of inflammatory cells, including monocytes, B- and T-lymphocytes, neutrophils and platelets play a critical role in the pathogenesis of this disease.<sup>4</sup> These cells exhibit pro-atherogenic functions through multiple co-stimulatory and immune molecules present on their cell surface. Among these, the CD40L/CD40 receptor-ligand pair has been the focus of much attention, such that this dyad is now regarded as a pivotal contributor to all underlying phases of atherothrombosis.<sup>5-7</sup> Indeed, the CD40L/CD40 interaction exerts a wide array of biological functions at the forefront of the pathophysiology of this disease and disruption of this cascade by both pharmacological and genetic approaches have shown beneficial results in animal and clinical studies.<sup>8-11</sup> While CD40L is known to mainly interact with its classical receptor CD40, additional binding partners have been described, namely the integrins  $\alpha_{IIb}\beta_3$ ,  $\alpha_M\beta_2$  and  $\alpha_5\beta_1$ . This chapter discusses the role of CD40L and its receptors in the pathophysiology of atherothrombosis, while highlighting its therapeutic potentials in the treatment of this chronic inflammatory disease.

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## 2. The CD40L system

### 2.1 CD40L

CD40L, also known as CD154, is a 39 kDa transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily originally identified on cells of the immune system.<sup>12, 13</sup> The interaction of CD40L with its respective receptor on B cells, CD40, a glycoprotein also from the TNF receptor (TNFR) family, is of critical importance for immunoglobulin isotype switching during the immune response.<sup>14</sup> The importance of this interaction is highlighted by the pathophysiological manifestations seen in patients suffering from the X-linked hyperimmunoglobulin-M syndrome, in which B-cells fail to produce the immunoglobulin's IgG, IgA and IgE as a consequence of a genetic mutation in the CD40L gene.<sup>15</sup> Because of its wide distribution across cells of the vascular system (endothelium, B and T lymphocytes, neutrophils, platelets, monocytes, dendritic cells and smooth muscle cells), the CD40L/CD40 dyad also shares important implications in cell-mediated immunity. CD40L-induced signaling in these cells leads to the up-regulation of adhesion and co-stimulatory molecules, and the production of pro-inflammatory cytokines, chemokines, growth factors, matrix metalloproteinases (MMPs) and procoagulants.<sup>16-19</sup> These cellular events are also the main mechanisms by which CD40L regulates numerous inflammatory disorders, in particular atherothrombosis and its related complications. In fact, circulating levels of soluble CD40L (sCD40L), which originate from the proteolytic cleavage of membrane-bound CD40L at the surface of activated platelets, have now emerged as strong indicators of cardiovascular events such as atherothrombosis and acute coronary syndromes.<sup>20-22</sup>

### 2.2 CD40L receptors

#### CD40

CD40 is the classical high affinity receptor for CD40L. It is constitutively or inducibly expressed by most cells of the vascular system (hematopoietic and non-hematopoietic cells) and represents the main signaling molecule in the CD40L/CD40 receptor-ligand pair.<sup>23</sup> The cytoplasmic domain of CD40 bears signaling domains required for the association of binding proteins termed TNF receptor-associated factors (TRAFs).<sup>24</sup> During humoral immunity, a tight interplay between dendritic cells, T-lymphocytes and B-lymphocytes occurs, throughout which the activation of CD40 provides a crucial signal for the activation, differentiation and secretion of immunoglobulins by B cells.<sup>25</sup> Moreover, CD40 activation in these cells induces an important anti-apoptotic signal that facilitates cell survival and differentiation, primarily through activation of the anti-apoptotic proteins Bcl-XL, A20, Bfl-1 and Mcl-1, which protect against Fas ligand and TNF-induced cell death.<sup>26</sup> As discussed above, CD40 signaling plays a significant role in cell-mediated immunity, an important aspect by which the CD40L/CD40 dyad initiates and exacerbates atherosclerotic lesions. For instance, CD40 activation on endothelial cells induces the up-regulation of a plethora of proinflammatory adhesion molecules, cytokines, chemokines, matrix metalloproteinases and procoagulants.<sup>16</sup> In addition, it has been demonstrated that upon CD40L binding CD40 activation on platelets can enhance platelet function and promote the secretion of inflammatory cytokines involved in plaque formation (this aspect will be discussed in greater detail below).<sup>27-29</sup>

### CD40-TRAF dependent signaling

The engagement of CD40 by CD40L promotes the clustering of CD40 and induces the association of TRAFs to the cytoplasmic domain of CD40.<sup>30</sup> These adapter proteins are essential for the activation of different signaling pathways including the canonical and non-canonical nuclear factor  $\kappa$ B (NF- $\kappa$ B)-signaling pathways and the activation of mitogen-activated protein kinases (MAPKs).<sup>30</sup> The TRAF family comprises six known members, among which TRAF-1, -2, -3, -5 and -6 have been shown to drive CD40-dependent cellular responses.

TRAF-1 can only bind weakly to the cytoplasmic tail of CD40 and therefore regulates the signaling of others TRAF members, in particular TRAF-2.<sup>30, 31</sup> Indeed, TRAF-1 deficiency in antigen presenting cells and B-lymphocytes leads to a significant reduction in the recruitment of TRAF-2 to CD40, indicating that TRAF-1 facilitates the association of TRAF-2 to the cytoplasmic domain of CD40.<sup>32, 33</sup> In agreement with these results, it has been shown that the recruitment of both TRAF-1 and TRAF-2 are required for complete activation of NF- $\kappa$ B in B-cells, since the knockout of both genes results in a greater inhibition of the NF- $\kappa$ B signaling pathway, in comparison to single knockouts.<sup>33</sup>

TRAF-2 is an important contributor to CD40 signaling and its major role resides in the activation of the NF- $\kappa$ B signaling pathway, as well as the activation of the p38, Akt and JNK MAPKs. CD40 bears a direct binding site for TRAF-2 and blockage of this interaction leads to immune deficiencies such as B-cell proliferation and isotype switching.<sup>24, 34</sup> Despite its significant implications in CD40 signaling, TRAF-2 deficiency may be overcome by TRAF-6 activation. This was confirmed by data showing that binding of either TRAF-2 or TRAF-6 alone may activate the NF- $\kappa$ B pathway, while inhibition of both these members completely abolishes CD40-dependant B-cell activation, suggesting that both members collaborate for the activation of this critical signaling cascade.<sup>35-37</sup>

TRAF-3 functions as a negative regulator of CD40 signaling through its constitutive association with TRAF-2.<sup>38, 39</sup> In absence of stimulation, TRAF-3 interacts with TRAF-2, which allows the degradation of the NF- $\kappa$ B inducing kinase (NIK) protein, a critical stimulator of NF- $\kappa$ B.<sup>40</sup> TRAF-3 deficiency in B cells exacerbates NF- $\kappa$ B and JNK activation, primarily through cytosolic accumulation of NIK, thus confirming the negative regulatory functions of TRAF-3 in B cells.<sup>41</sup>

Very little information is available regarding the role of TRAF-5 in CD40 signaling. Nevertheless, it appears that TRAF-5 can interact with TRAF-3 to modulate NF- $\kappa$ B activation in B cells. This was shown by experiments in which TRAF-5 deficiency diminishes NF- $\kappa$ B activation, causing a reduction in cell activation, expression of co-stimulatory molecules and antibody production.<sup>42, 43</sup>

TRAF-6 plays a significant role in the activation of key CD40-dependent signaling pathways, such as NF- $\kappa$ B, p38, JNK and Akt.<sup>44</sup> As discussed above, TRAF-6 synergizes with TRAF-2 in order to regulate the activation of NF- $\kappa$ B. Although TRAF-6 contains a direct binding site for CD40, specific inhibition of this domain shows lesser inhibitory effects than ablation of the complete protein, indicating that TRAF-6 may still have a functional role in CD40 signaling without binding directly to CD40.<sup>30, 45</sup> Indeed, one of the main functions of TRAF-6 resides in its ability to interact with TRAF-2, which is already bound to CD40, and facilitate the activation of downstream targets.

Recently, a study aiming at evaluating the implication of TRAF members in neointima formation, a critical step of atherothrombosis, was conducted. Using a CD40 transgenic mouse model, in which mutations at the TRAF2/3/5, TRAF6 or TRAF2/3/5/6 binding sites were carried out, the authors conclude that the CD40-TRAF6 axis is a key regulator of inflammatory cell infiltration and neointima formation at sites of vascular injury.<sup>46</sup>

Although most vascular complications associated to CD40L, including atherothrombosis, have been largely attributed to its interaction with CD40, recently identified additional receptors merit attention. These include the integrins  $\alpha_{IIb}\beta_3$ ,  $\alpha_5\beta_1$  and  $\alpha_M\beta_2$  and (Figure 1).

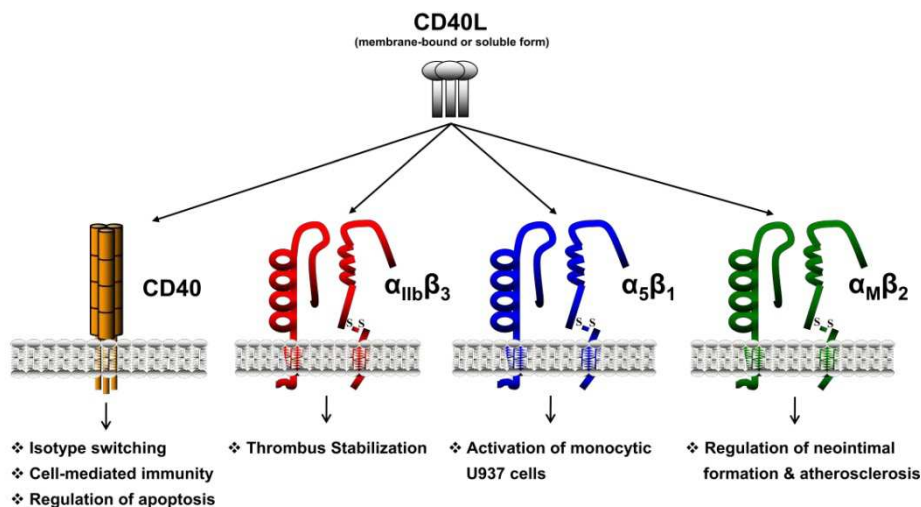


Fig. 1. CD40L and its receptors. The binding of CD40L to its classical CD40 counterreceptor regulates numerous critical biological responses. These mainly include B-cell dependent isotype switching, cell-mediated immunity (production of cytokines, chemokines, adhesion molecules, growth factors, MMPs and procoagulants) and apoptosis. The CD40L/CD40 interaction is at the forefront of the pathogenesis of multiple inflammatory disorders, including atherothrombosis. The interaction of CD40L with the  $\alpha_{IIb}\beta_3$  platelet integrin is involved in thrombus stabilization and may provide a novel outside-in signaling pathway by which platelets can be activated. CD40L can also bind to the inactive conformation  $\alpha_5\beta_1$  and this interaction was shown to induce activation of the human monocytic U937 cell line. Finally,  $\alpha_M\beta_2$  can mediate CD40L-dependent inflammatory responses, in particular leukocyte adhesion and neointimal formation. The pathophysiological relevance of these novel CD40L-mediated interactions in inflammation remains elusive and additional studies will be required to address this issue.

### $\alpha_{IIb}\beta_3$

The  $\alpha_{IIb}\beta_3$  integrin is the most abundant receptor of the surface of platelets and mediates platelet adhesion and aggregation. Like all molecules of the integrin family, it will change conformation upon inside-out cellular activation, thereby allowing binding to its natural ligands (fibrinogen, fibronectin, vWF...).<sup>47</sup> These ligands contain KGD sequences and

binding is mediated through the KGD recognition domain present on the  $\alpha\text{IIb}\beta 3$  molecule. Interestingly, CD40L also contains a KGD sequence making its interaction with  $\alpha\text{IIb}\beta 3$  possible. Binding of CD40L to  $\alpha\text{IIb}\beta 3$  was shown to induce phosphorylation of tyrosine residues within the cytoplasmic domain of the  $\beta 3$  subunit and appears essential for thrombus stabilization *in vivo*.<sup>48, 49</sup> Indeed, CD40L<sup>-/-</sup> mice exhibit unstable thrombi, which can be overcome by infusion of wild type recombinant human CD40L and not CD40L specifically mutated at the site of interaction with  $\alpha\text{IIb}\beta 3$ .<sup>48</sup>

### $\alpha 5\beta 1$

The  $\alpha 5\beta 1$  integrin was also shown to act as a CD40L receptor.<sup>50</sup> Indeed, sCD40L can bind and activate cells of the undifferentiated human monocytic U937 cell line in a CD40- and  $\alpha\text{IIb}\beta 3$ -independent manner. Binding to this cell line was reversed by an anti- $\alpha 5\beta 1$  antibody, as well as in the presence of soluble  $\alpha 5\beta 1$ , thus confirming  $\alpha 5\beta 1$  specificity. Moreover, this interaction is unaffected by pre-treatment of CD40L with soluble CD40, indicating that CD40L can bind both CD40 and  $\alpha 5\beta 1$  concomitantly.<sup>50</sup> Interestingly, CD40L binds to inactive  $\alpha 5\beta 1$ , contrary to most ligands of the integrin family. However, the physiological relevance of this interaction remains unexplored and additional studies are needed to fully characterize the interplay that might take place between CD40L and  $\alpha 5\beta 1$  in inflammatory disorders.

### $\alpha\text{M}\beta 2$

The  $\alpha\text{M}\beta 2$  (Mac-1) integrin mediates firm adhesion of leukocytes to inflamed vessels by interacting with its endothelial cell counterreceptor intercellular adhesion molecule 1 (ICAM-1).<sup>51</sup> CD40L was also recently shown to bind to active  $\alpha\text{M}\beta 2$  and this interaction may represent an alternative pathway for CD40L-mediated inflammation.<sup>52</sup> Indeed, inhibition of this novel CD40L binding partner significantly attenuates leukocyte accumulation at sites of inflammation and reduces atherogenesis, indicating that CD40L may promote, at least in part, atherosclerotic lesions in a  $\alpha\text{M}\beta 2$ -dependent manner. Again, the relative contribution of this CD40L receptor (in comparison to CD40) in the development of inflammatory disorders such as atherothrombosis remains unknown. Perhaps, each of these CD40L receptors may interfere at different stages of the disease, thus contributing to proinflammatory reactions and atherogenesis in their own way.

## 3. CD40L in atherothrombosis

The involvement of CD40L in the pathogenesis of atherothrombosis is supported by numerous studies. Targeting of CD40L by both pharmacological and genetic approaches has highlighted the importance of this molecule in all stages of the disease. In 1998, Mach et al. showed that treatment of hyperlipidemic LDLR<sup>-/-</sup> mice with an anti-CD40L antibody significantly ameliorates the size and lipid contents of atherosclerotic lesions.<sup>53</sup> These results were further confirmed by a genetic approach, which showed that CD40L<sup>-/-</sup>/ApoE<sup>-/-</sup> mice exhibit considerably smaller plaque area than control ApoE<sup>-/-</sup> mice.<sup>8</sup> Moreover, these animals display enhanced collagen fibrils within the fibrous cap of lesions, a key component of plaque stability. In an additional study, the administration of an anti-CD40L antibody in ApoE<sup>-/-</sup> mice, at the onset of lesions or once atherosclerotic lesions are fully established, reduces lipid contents and increases plaque stability.<sup>54</sup> Taken together, these studies support the contribution of CD40L in plaque initiation, progression and stability (Figure 2).

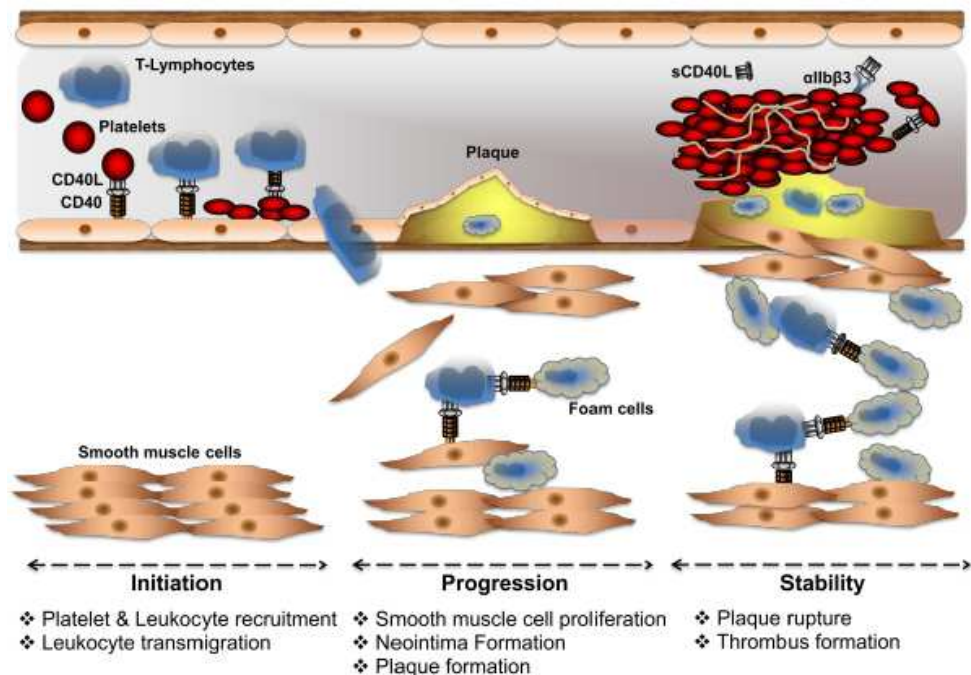


Fig. 2. Role of CD40L in atherothrombosis. The incorporation of oxidized LDLs, among other factors, may upregulate the expression of the CD40L system on the endothelium, thereby promoting the recruitment of platelets and leukocytes at the sites of injury. The CD40L-dependent adhesion of T-lymphocytes and platelets to the endothelium induces an important inflammatory response characterized by the secretion of various cytokines and the upregulation of additional endothelial adhesion molecules. This favors in turn the incorporation and transmigration of additional leukocytes, in particular monocytes. Once in the sub-endothelial space, CD40L/CD40 interactions between foam cells (macrophages which have undergone phagocytosis of oxidized LDL particles), T-lymphocytes and smooth muscle cells take place. These cross talks ultimately lead in part to the proliferation of smooth muscle cells into the intima and promote vascular angiogenesis, primarily through the secretion of key inflammatory and angiogenic cytokines and chemokines. This process eventually yields a stable lipid-enriched atherosclerotic plaque surrounded by a fibrous cap. Plaque stability is threatened by the production of MMPs, which are directly responsible for collagen degradation and rupture of the fibrous cap. The binding of CD40 on endothelial cells, macrophages and smooth muscle cells can provoke the secretion of a long list of MMPs. Following rupture, platelets rapidly adhere to the surface of the highly prothrombotic contents of the atherosclerotic plaque, thereby leading to thrombus formation and arterial occlusion. CD40L may also be involved in thrombus formation and procoagulant activity. CD40L binding to the endothelium promotes tissue factor expression, while the binding of CD40L (soluble and membrane-bound forms) to CD40 and  $\alpha\text{IIb}\beta\text{3}$  on platelets enhances platelets aggregation and thrombus stabilization, respectively.

### 3.1 Initiation of lesions and leukocyte recruitment

Plaque initiation is normally characterized by the accumulation of low-density lipoproteins (LDL) in the arterial wall and the subsequent recruitment and transmigration of leukocytes within the sub-endothelial space.<sup>1</sup> The initial trigger of CD40L (and CD40) expression within cells of the developing atherosclerotic plaque (endothelial cells, lymphocytes, platelets, monocytes/macrophages, and smooth muscle cells) remains elusive. Possible candidates include oxidized LDL, infectious pathogens and alterations in vascular hemodynamic forces.<sup>55-57</sup> For instance it has been demonstrated that lipid lowering reduces CD40L expression in atheroma.<sup>55</sup> In addition, oxidized LDL were reported to induce the expression of CD40 on endothelial cells, which can then bind CD40L from activated T-lymphocytes adherent to the site of injury.<sup>58</sup> CD40 activation on endothelial cells provides a critical proinflammatory signal for the initiation of lesions. Indeed, the CD40L/CD40 interaction favors the up-regulation of adhesion molecules (E-selectin, P-selectin, vascular cell adhesion molecule-1 [VCAM-1] and ICAM-1) and leads to the secretion of proinflammatory cytokines (IL-6, IL-8, IL-15, monocytes chemotactic protein-1 [MCP-1], macrophage inflammatory protein-1 [MIP-1  $\alpha/\beta$ ] and regulated on activation normal T cell expressed and secreted [RANTES]) by the endothelium.<sup>6, 7, 59-62</sup> These reactions induce in turn the incorporation and accumulation of additional leukocytes, in particular monocytes, at the sites of developing lesions.

As discussed above,  $\alpha\text{M}\beta\text{2}$ , an integrin expressed on neutrophils and monocytes/macrophages, has been identified as a receptor for CD40L. This interaction may also mediate adhesion and migration of inflammatory cells at sites of plaque initiation. In agreement with this hypothesis,  $\alpha\text{M}\beta\text{2}$  deficiency attenuates lesion development and reduces lesional macrophage accumulation in LDLR<sup>-/-</sup> mice, supporting the implication of this integrin in atherothrombosis.<sup>52</sup> However, additional studies will be required to specifically establish the importance of the CD40L/ $\alpha\text{M}\beta\text{2}$  in plaque initiation.

In addition, platelets have been shown to play a crucial role in the initiation of atherothrombosis. Platelets are among the first inflammatory cells at the site of injury and their adhesion to the endothelium provides a fundamental mechanism by which leukocytes are recruited.<sup>63, 64</sup> Because the surface of activated platelets contains a higher density of P-selectin than activated endothelial cells, significantly more leukocytes will incorporate at the sites of injury in their presence.<sup>65</sup> Interestingly, CD40L from activated platelets can also induce a proinflammatory response on endothelial cells, in a similar fashion to that of T-lymphocytes. Henn et al. demonstrated that CD40 ligation on endothelial cells by CD40L from activated platelets induces the expression of numerous adhesion molecules, cytokines, and matrix metalloproteinases involved in the initiation of inflammatory reactions.<sup>16</sup>

### 3.2 Plaque development and progression

The progression of the atherosclerotic plaque is typically highlighted by the proliferation and migration of smooth muscle cells into the intima, as well as the formation neovessels (angiogenesis), which supports the growth of lesions. This process will eventually yield a stable lipid-enriched necrotic plaque surrounded by a fibrous cap. Once in the sub-endothelial space, macrophages (originally monocytes) undergo phagocytosis of oxidized LDL particles, leading to the formation of foam cells.<sup>66</sup> Thereafter, CD40L from infiltrated T-

lymphocytes will bind to CD40 on the surface of differentiated foam cells, favoring the release of further proinflammatory cytokines (IL-1, IL-6 and IL-12), growth factors (vascular endothelial growth factor [VEGF]) and MMPs (MMP-1 and MMP-3).<sup>7, 67</sup> These responses are intimately involved in the proliferation and migration of smooth muscle cells into the intima layer. In parallel, cross talks between smooth muscle cells and T-lymphocytes may also take place, in which CD40 activation on the former initiates a positive feedback loop enhancing the inflammatory reactions already in place.<sup>6</sup> Indeed, CD40 signaling in smooth muscle cells has been shown to induce the secretion of the cytokines IL-8 and MCP-1.<sup>67, 68</sup> Moreover, the accumulation of migrating fibroblasts within the intima layer exacerbates the atherosclerotic lesions in development and the CD40L/CD40 axis might also take part in this process.<sup>69</sup> Stimulation of fibroblasts with CD40L was reported to up-regulate the expression of cell surface adhesion molecules, thus facilitating their interaction with immune cells at the site of lesions.<sup>70</sup> This interaction also induces their proliferation and secretion of chemoattractant cytokines such as IL-6 and IL-8.<sup>71-73</sup> Hence, the CD40L/CD40 interaction is at the forefront of a plethora of key inflammatory reactions involved in neointima formation and plaque accumulation. Lesions with eventually develop into the formation of a stable necrotic core consisting of infiltrated leukocytes, foam cells, proliferating smooth muscle cells, extracellular matrix proteins and lipids.

The formation of neovessels or angiogenesis plays an integral part in plaque progression and several reports have highlighted the importance of CD40L in this process. For instance, CD40 ligation on endothelial cells and macrophages was shown to upregulate the expression of potent angiogenic factors such as VEGF, fibroblast growth factor and platelet-activating factor, in addition to inducing the synthesis and proteinase activity of various MMPs such as MMP-1, MMP-2, MMP-3 and MMP-9.<sup>18, 74-76</sup> These responses are tightly linked to tubule formation and angiogenesis, essential elements of plaque support and growth. Interestingly, the  $\alpha 5\beta 1$  integrin is upregulated on angiogenesis-prone endothelial cells and could also provide a novel mechanism by which CD40L modulates pathological angiogenesis.<sup>77</sup> It would be worthwhile investigating this issue in further details.

### 3.3 Plaque instability and thrombosis

Plaque stability is regulated by a tight balance between extracellular matrix proteins such as collagen fibers and MMP production. A thin fibrous cap protects the highly thrombotic components of the atherosclerotic plaque. However, upon secretion of MMPs by macrophages and other inflammatory cells present, plaque rupture may ensue following digestion of the collagen fibers within the fibrous cap.<sup>78, 79</sup> This process leads to thrombus formation and may cause complete obstruction of the artery. CD40L mediates several of the processes that set the stage for plaque rupture and its clinical sequelae. CD40L stimulation on endothelial cells, macrophages and smooth muscle cells can provoke the secretion of a long list of MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13), the main digestive enzymes of the collagen-rich fibrous cap.<sup>18, 80, 81</sup> Platelets, in addition to their pivotal role in thrombosis, also participate in this process. Indeed, membrane-bound CD154 expressed on the surface of activated platelets can induce MMP upregulation in endothelial cells.<sup>82</sup> MMP secretion and proteolytic activity can be abrogated by physical hindrance of platelet-endothelial contacts,  $\alpha \text{IIb}\beta 3$  interfering agents or anti-CD40L antibodies, thus highlighting in part the importance of platelets and CD40L in this phenomenon.



Following rupture, platelets rapidly adhere to the surface of the highly pro-thrombotic contents of the atherosclerotic plaque, thereby leading to thrombus formation and arterial occlusion.<sup>83, 84</sup> Accumulating evidence also support a role for CD40L in platelet function and thrombus formation, albeit some of the data remain conflicting. For instance, Andre et al. have shown that CD40L plays a role in thrombus stabilization by interacting with  $\alpha$ Ib $\beta$ 3, while we and others have demonstrated that CD40L enhances platelet aggregation and thrombus formation through a CD40-mediated TRAF-2/Rac1/p38 signaling pathway.<sup>27, 28, 48</sup> Indeed, enhanced levels of circulating sCD40L exacerbate thrombus formation *in vivo*, also in a CD40-dependent fashion.<sup>27</sup> Nevertheless, these studies all support the concept of CD40L as a pro-thrombotic agent, predisposing platelets to enhanced cell function. CD40L may also enhance the coagulation system through the induction of tissue factor release from various vascular cells. CD40 engagement on endothelial cells, macrophages and smooth muscle cells by CD40L from activated platelets or T cells induces tissue factor expression and activity.<sup>85-87</sup> Besides its important role in the induction of the extrinsic coagulation cascade, tissue factor also represents a powerful platelet agonist.

#### 4. Soluble CD40L and coronary syndromes

Given the pivotal contribution of the CD40L system in atherothrombosis, multiple clinical studies have evaluated the association between levels of circulating sCD40L and cardiovascular risk, in particular acute coronary syndrome (ACS) such as acute myocardial infarction (AMI) and unstable angina (UA). These studies can be divided into two main categories. The first type of clinical studies has investigated the link between levels of sCD40L and ACS, while the second has determined the link between levels of sCD40L and prognosis and risk prediction.

For the most part, clinical studies demonstrate that circulating sCD40L levels are significantly higher in patients with ACS and stable coronary artery disease (CAD), compared with control subjects.<sup>88-92</sup> Indeed, it appears that a gradual increase in sCD40L levels occurs with ACS progression, with peaks as early as 9 hours following the onset of AMI or UA.<sup>89, 93, 94</sup> For instance, patients suffering from AMI or UA present with levels ranging from 5-25 ng/mL, depending on the study.<sup>88</sup> Moreover, sCD40L levels are independent from other important inflammatory markers, such as IL-6, sICAM-1, sVCAM-1, C-reactive protein and troponin, indicating that sCD40L may represent a more reliable risk factor, as compared to others.<sup>92</sup> Because sCD40L in circulation almost exclusively originates from the shedding of membrane-bound CD40L at the surface of activated platelets, its measuring levels may reflect a state of platelet activation rather than an inflammatory condition.

More importantly, some clinical studies have evaluated the relationship between sCD40L levels and disease prognosis. In the CAPTURE (c7E3 Fab Anti-Platelet Therapy in unstable Refractory Angina) trial, patients with high levels of sCD40L were 3-fold more at risk of developing cardiovascular death or AMI.<sup>21</sup> Moreover, in the MIRACL (Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering) study, sCD40L levels were an independent risk factor for recurrent cardiovascular events, such as death, nonfatal myocardial infarction, cardiac arrest and worsening angina requiring rehospitalization.<sup>95</sup> Interestingly, individuals carrying the -3459A>G polymorphism on the CD40L gene, are more at risk of developing AMI.<sup>96</sup>

Whether enhanced levels of sCD40L seen in patients with ACS are a consequence of increased platelet activation or a predetermining cause of these complications (or perhaps both) is still unknown. Recently, we have shown that enhancing levels of circulating sCD40L in mice to approximately 45 ng/mL exacerbates thrombus formation in a CD40-dependent manner.<sup>27</sup> This observation supports the idea that increased levels of sCD40L in patients may drive, at least in part, the development of certain cardiovascular complications. It would be tempting to speculate the existence of a positive feedback loop taking place in these patients, where disease initiation correlates with platelet activation and release of sCD40L in the circulation. This in turn could further exacerbate pre-existing complications through enhancement of platelet function and thrombus formation.

## **5. Disruption of the CD40L system as a therapeutic target in atherothrombosis**

In light of all the aforementioned data supporting the contribution of CD40L in inflammation, disruption of this system as a therapeutic strategy for the treatment of atherothrombosis and its clinical manifestations has been investigated. Unfortunately, clinical trials using an anti-CD40L antibody were put on hold due to thromboembolic complications.<sup>97, 98</sup> Interactions between CD40 and CD40L-immune complexes at the surface of platelets have been suggested as a possible mechanism by which CD40L therapy induces these complications.<sup>97</sup>

Since circulating levels of sCD40L result from platelet activation, indirect targeting of the CD40L system through anti-platelet therapy may represent an alternative approach to suppress this important component. Clopidogrel, a potent inhibitor of the platelet adenosine diphosphate (ADP) receptor, has been reported to block sCD40L release from ADP-stimulated platelets.<sup>99</sup> Interestingly, clopidogrel regimen significantly reduces platelet CD40L expression and sCD40L levels in patients with stable CAD.<sup>100</sup> Moreover,  $\alpha$ IIb $\beta$ 3 inhibitors, such as abciximab, inhibit platelet aggregation and sCD40L release from activated platelets.<sup>101</sup> In the CAPTURE trial, abciximab significantly reduces sCD40L levels and cardiovascular risk in high-risk ACS patients, confirming a link between  $\alpha$ IIb $\beta$ 3 signaling and platelet sCD40L release.<sup>21</sup>

Statins exert multiple pleiotropic anti-inflammatory effects, in addition to their lipid lowering properties. Several reports have investigated the effects of these drugs on inflammatory markers, including CD40L. Particularly, they have been shown to reduce cytokine-induced CD40L expression on endothelial cells, smooth muscle cells and macrophages.<sup>102</sup> Notably, atorvastatin treatment in the MIRACL trial reduces the risk of recurrent cardiovascular events, which are associated with sCD40L levels.<sup>95</sup>

Most of these agents indirectly target the CD40L system, perhaps through inhibition of platelet activation and the subsequent release of sCD40L. Specific disruption of CD40L or its receptors remains a promising approach for the treatment of atherothrombosis. Although clinical studies using anti-CD40L antibodies have been unsuccessful, alternative targets of this system may render better clinical outcomes. For example, novel anti-CD40L agents that specifically target the interaction of CD40L with its different receptors or inhibition of critical intracellular signaling elements, such as TRAFs, represent valuable approaches.

## 6. Conclusions

Research over the years overwhelmingly supports the notion of atherothrombosis as a chronic inflammatory disease. Despite the plethora of inflammatory mediators identified thus far as potential contributors to this complication, the CD40L system has attracted a great deal of interest. Besides its pivotal role in humoral immunity, CD40L is now regarded as a key player to all major phases of atherothrombosis, a concept supported in part by the strong relationship between its circulating soluble levels and the occurrence of cardiovascular diseases. In addition to its well-established CD40 counterreceptor, CD40L can also interact with novel binding partners, namely the integrin receptors  $\alpha_{IIb}\beta_3$ ,  $\alpha_M\beta_2$  and  $\alpha_5\beta_1$ . Although most CD40L-mediated functions have been attributed to its interaction with CD40, these novel receptors add complexity to the diverse interplays that might take place during inflammation. The elucidation of the exact physiopathological relevance of these interactions in inflammatory disorders might pave the way for the development of novel anti-CD40L therapeutic targets for the treatment of atherothrombosis and cardiovascular diseases.

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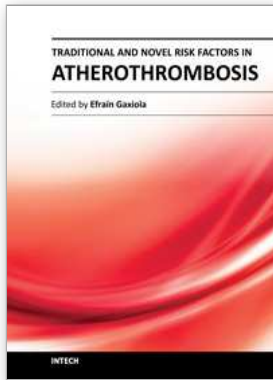
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## **Traditional and Novel Risk Factors in Atherothrombosis**

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Atherothrombosis has reached pandemic proportions worldwide. It is the underlying condition that results in events leading to myocardial infarction, ischemic stroke and vascular death. As such, it is the leading cause of death worldwide manifested mainly as cardiovascular/cerebrovascular death. The complex and intimate relationship between atherothrombosis and traditional and novel risk factors is discussed in the following chapters of Traditional and Novel Risk Factors in Atherothrombosis - from basic science to clinical and therapeutic concerns. Beginning with pathology and pathophysiology of atherothrombosis, plaque rupture/disruption, this book continues with molecular, biochemical, inflammatory, cellular aspects and finally analyzes several aspects of clinical pharmacology.

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