Enhanced Levels of Soluble CD40 Ligand Exacerbate Platelet Aggregation and Thrombus Formation Through a CD40-Dependent Tumor Necrosis Factor Receptor–Associated Factor-2/Rac1/p38 Mitogen-Activated Protein Kinase Signaling Pathway*

Daniel Yacoub, Ahmed Hachem, Jean-François Théorêt, Marc-Antoine Gillis, Walid Mourad, Yahye Merhi

Objective—CD40 ligand is a thromboinflammatory molecule that predicts cardiovascular events. Platelets constitute the major source of soluble CD40 ligand (sCD40L), which has been shown to influence platelet activation, although its exact functional impact on platelets and the underlying mechanisms remain undefined. We aimed to determine the impact and the signaling mechanisms of sCD40L on platelets.

Methods and Results—sCD40L strongly enhances platelet activation and aggregation. Human platelets treated with a mutated form of sCD40L that does not bind CD40, and CD40−/− mouse platelets failed to elicit such responses. Furthermore, sCD40L stimulation induces the association of the tumor necrosis factor receptor–associated factor-2 with platelet CD40. Notably, sCD40L primes platelets through activation of the small GTPase Rac1 and its downstream target p38 mitogen-activated protein kinase, which leads to platelet shape change and actin polymerization. Moreover, sCD40L exacerbates thrombus formation and leukocyte infiltration in wild-type mice but not in CD40−/− mice.

Conclusion—sCD40L enhances agonist-induced platelet activation and aggregation through a CD40-dependent tumor necrosis factor receptor–associated factor-2/Rac1/p38 mitogen-activated protein kinase signaling pathway. Thus, sCD40L is an important platelet primer predisposing platelets to enhanced thrombus formation in response to vascular injury. This may explain the link between circulating levels of sCD40L and cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2010;30:2424-2433.)

Key Words: platelets ■ signal transduction ■ thrombosis ■ CD40L

Multiple lines of evidence now support a plethora of inflammatory mediators potentially involved in the pathogenesis of vascular disease. Among these, the CD40 ligand (CD40L)/CD40 dyad has been the focus of much attention, and circulating levels of soluble CD40L (sCD40L) are now considered reliable predictors of cardiovascular events.1−4

CD40L is a 48-kDa trimeric transmembrane protein belonging to the tumor necrosis factor superfamily originally identified on cells of the immune system.5,6 Interaction of CD40L with its respective receptor on B cells, CD40, a 39-kDa glycoprotein from the tumor necrosis factor receptor family, is of critical importance for immunoglobulin isotype switching during the immune response.7 Today, we know that these 2 molecules are also present on cells of the vascular system, including endothelial cells, monocytes/macrophages, smooth muscle cells, and platelets,8,9 and have important implications in inflammatory reactions, through upregulation of cell adhesion molecules and production of proinflammatory cytokines, chemokines, growth factors, matrix metalloproteinases, and procoagulants.8,10−12 The involvement of the CD40L/CD40 dyad in thromboinflammation has been highlighted in all pathogenic phases of atherosclerosis, including endothelial dysfunction, platelet activation, thrombosis, and neointima formation.13−20

Platelets are highly specialized blood cells of paramount importance in normal hemostasis and thromboinflammatory complications. The pioneering work of Henn et al showed that both CD40L and its receptor CD40 are found in platelets.8 Whereas CD40 is constitutively expressed on platelets, CD40L rapidly appears on the platelet surface following

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From the Montreal Heart Institute, Montreal, Quebec, Canada (D.Y., A.H., J.-F.T., M.-A.G., Y.M.); Faculty of Medicine (D.Y., A.H., J.-F.T., M.-A.G., Y.M.) and Research Centre, Centre Hospitalier de l’Université de Montréal (CHUM) (W.M.), Université de Montréal, Montreal, Quebec, Canada.
D. Yacoub and A. Hachem contributed equally to this work.
*Original research submitted in response to ATVB’s call for papers.
Correspondence to Yahye Merhi, PhD, Laboratory of Thrombosis and Hemostasis, Montreal Heart Institute, 5000 Belanger, Montreal, Quebec H1T 1CS, Canada. E-mail yahye.merhi@icm-mhi.org
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Figure 1. sCD40L enhances platelet activation and aggregation through interaction with CD40. A, Dose-dependent effect of sCD40L on platelet aggregation. Platelets were preincubated with the indicated concentrations of sCD40L for 30 minutes at 37°C, and aggregation was induced by a priming dose of collagen (0.25 μg/mL). Upper aggregation traces show the effect of sCD40L alone (1 μg/mL). Histogram represents the mean of data of platelet aggregation in response to sCD40L alone (no agonist) or in the presence of a priming (low) dose of collagen (0.25 μg/mL), thrombin (0.02 U/mL), or ADP (5 μmol/L). High doses of agonists (5 μg/mL collagen, 0.5 U/mL thrombin, or 20 μmol/L ADP) were used as positive controls (n=5; *P<0.05 versus low dose). B, Effect of sCD40L on P-selectin (CD62P) expression and αIIbβ3 activation, as assessed by flow cytometry. Left plots represent resting platelets (−collagen: gray for baseline, black for sCD40L). Right plots represent platelets in the presence of a priming concentration of collagen (+collagen: gray for baseline, black for sCD40L).
activation, on which it is subsequently cleaved, generating a soluble fragment of 18-kDa, termed sCD40L, accounting for >95% of its plasmatic concentration.\textsuperscript{21} Circulating levels of sCD40L in patients have now emerged as strong indicators of cardiovascular risk, as there appears to be a significant correlation between levels of sCD40L and vascular complications such as atherosclerosis and acute coronary syndromes (ACS).\textsuperscript{2-4}

Although the presence of the CD40L/CD40 dyad in platelets is well defined, its exact involvement in platelet function remains elusive. Andre et al have shown that CD40L binds to $\alpha_{IIb}\beta_3$ and stabilizes arterial thrombi in mice,\textsuperscript{13} whereas others have shown that CD40L can induce platelet activation and secretion of reactive oxygen species and the chemokine RANTES through binding of CD40.\textsuperscript{14,17,22} Nevertheless, the physiological impact and the mechanisms involved in CD40L-induced platelet activation are poorly characterized. Here we show that sCD40L primes and enhances agonist-induced activation and aggregation of human platelets through a CD40-mediated tumor necrosis factor receptor–associated factor (TRAF)-2/Rac1/p38 mitogen-activated protein kinase (MAPK)–dependent pathway, which ultimately leads to platelet shape change and actin polymerization. Moreover, we show that enhanced levels of sCD40L exacerbate thrombus formation and leukocyte infiltration in response to vascular injury, in a CD40-dependent manner.

Materials and Methods

An expanded Methods section is available in the supplemental materials, available online at http://atvb.ahajournals.org.

This study has been approved by the human and animal ethical committees of the Montreal Heart Institute. All human subjects gave informed consent and were free from drugs interfering with platelet function. This study was conducted with washed human platelets and platelets from wild-type (WT) and CD40$^{-/-}$ mice. Platelet aggregation was assessed optically, and activation was determined by flow cytometry. We used a commercial form of sCD40L (R&D Systems), as well as a recombinant mutated form of sCD40L that does not recognize CD40 (sCD40L$^{\text{WT}}$), and its WT counterpart. Expression of TRAFs in total platelet lysates and in CD40 immunoprecipitates was determined by SDS-PAGE. Platelet shape change and actin polymerization were analyzed by scanning electron and confocal microscopy, respectively. Rac1 activation was assessed by a p21-activated kinase 1-PBD pull-down assay of its GTP-bound form, and vasodilator-stimulated phosphoprotein (VASP) and p38 MAPK activation were measured by SDS-PAGE using specific antibodies against the phosphorylated form of VASP on Ser157 and p38 MAPK on Thr180/Tyr182. In vivo thrombosis was measured in a FeCl3–induced carotid injury model in WT and CD40$^{-/-}$ mice. Leukocyte accumulation at the sites of injury was measured by CD45 immunostaining of thrombus sections.

Results

sCD40L Enhances Platelet Activation and Aggregation Through Interaction With CD40

We first evaluated the functional effects of sCD40L on platelet activation and aggregation, as it remains poorly characterized. Incubation of platelets with sCD40L alone had no effect on platelet aggregation (Figure 1A, upper panel) but led to a significant and dose-dependent increase of platelet aggregation induced by a subthreshold or priming concentration of collagen, thrombin, or ADP (Figure 1A), indicating that this is a broad platelet phenomenon and not agonist-specific. As sCD40L showed a significant impact on platelet aggregation, we sought to determine its effect on platelet activation. As expected, sCD40L was unable to trigger activation of $\alpha_{IIb}\beta_3$ on resting platelets, whereas it caused a significant increase in P-selectin expression (Figure 1B). However, $\alpha_{IIb}\beta_3$ activation and P-selectin expression were both significantly enhanced in the presence of subthreshold concentrations of collagen, thrombin, and ADP.

Because it has been shown that $\alpha_{IIb}\beta_3$, in addition to CD40, can constitute a CD40L receptor on the platelet surface,\textsuperscript{13,23} it was imperative to investigate through which receptor sCD40L acts. To address this issue, we first generated a mutant recombinant sCD40L (sCD40L$^{\text{WT}}$) that does not bind CD40 while retaining $\alpha_{IIb}\beta_3$ binding (Supplemental Figure I) and showed that this molecule failed to enhance platelet aggregation induced by a priming concentration of collagen, compared with its WT counterpart (sCD40L$^{\text{WT}}$) (Figure 1C). Second, unlike platelets from WT mice, platelets from CD40$^{-/-}$ mice were insensitive to mouse sCD40L in response to a subthreshold concentration of collagen (Figure 1D). These data clearly show that sCD40L enhances platelet function through interaction with its counterreceptor CD40.

sCD40L Triggers TRAF-2 Association With CD40

It is well established that the TRAF family is tightly linked to CD40 signaling in immune cells. The TRAF family comprises 6 known members, among which TRAF-1, -2, -3, and -6 have been the most studied, although their expression in platelets is yet to be determined. We therefore investigated the expression of the major TRAF members and their association with CD40 on sCD40L stimulation. Interestingly, we found that in addition to CD40, platelets express TRAF-1, -2, and -6, and traces of TRAF-3 were detected (Figure 2A). Most importantly, only TRAF-2 associates with CD40 after stimulation of resting platelets with sCD40L, whereas none of these members were shown to be associated with CD40 at

Figure 1 (Continued). Priming dose of collagen, black for priming dose + sCD40L, and white for high-dose collagen alone, as positive control. Histograms represent the mean of data of plots for CD62P expression and $\alpha_{IIb}\beta_3$ activation in response to sCD40L alone (no agonist) or in the presence of a priming (low) dose of collagen (1 $\mu$g/mL), thrombin (0.02 U/mL), or ADP (5 $\mu$mol/L). High doses of agonists (5 $\mu$g/mL collagen, 0.5 U/mL thrombin, or 20 $\mu$mol/L ADP) were used as positive controls ($n=5$; \(P<0.05\) versus baseline and \(P<0.05\) versus low dose). C, Effect of recombinant mutant sCD40L$^{\text{WT}}$ and sCD40L$^{\text{WT}}$ on human platelet aggregation. Platelets were incubated with 1 $\mu$g/mL of sCD40L$^{\text{WT}}$ or sCD40L$^{\text{WT}}$ for 30 minutes at 37°C and aggregation was induced by a priming dose of collagen (0.25 $\mu$g/mL). sCD40L was used as a positive control. D, Effect of mouse (m) sCD40L on WT and CD40$^{-/-}$ mouse platelet aggregation. Platelets were incubated with msCD40L (1 $\mu$g/mL) for 30 minutes at 37°C, and aggregation was induced by a priming concentration of collagen (1 $\mu$g/mL). Data in parentheses shown in C and D represent the mean±SEM of \(n=4\).
baseline conditions (Figure 2A and 2B). In addition, sCD40LWT, but not sCD40L R/Y, caused association of TRAF-2, further supporting the contribution of CD40 in response to sCD40L.

sCD40L Induces Platelet Shape Change and Actin Polymerization

In search of the underlying cellular and molecular events involved in the effects of sCD40L on platelet function, we first found that stimulation of resting platelets with sCD40L caused a significant morphological shape change characterized by an increase in lamellipodia and filopodia formation (Figure 3A). Second, sCD40L induced actin polymerization, as noted by the increase in F-actin staining in treated platelets (Figure 3B). To determine the implication of these cytoskeletal and morphological changes in sCD40L-induced potentiation of platelet aggregation, we pretreated platelets with latrunculin B, a specific inhibitor of actin polymerization, and found that it completely reversed sCD40L’s capacity to increase platelet aggregation (Figure 3C and 3D).

The Small GTPase Rac1 and p38 MAPK Are Required for sCD40L Signaling

The Rho family GTPase member Rac1 and the VASP represent key signaling components required for cytoskeletal reorganization and shape change in platelets. Phosphorylation of VASP, particularly at Ser157, regulates its anticaeping activity and thereby promotes platelet filopodia formation.24 As shown in Figure 4A, stimulation of resting platelets with sCD40L, but not with sCD40L R/Y, induced phosphorylation of VASP on Ser157 and activation of Rac1. To assess the implication of the small GTPase Rac1, we used a specific Rac1 inhibitor, NSC23766. Pretreatment of platelets with NSC23766 significantly reversed sCD40L’s ability to enhance platelet aggregation (Figure 4B) and P-selectin expression (Figure 4C) in resting platelets.

Activation of p38 MAPK is of significant importance in CD40 signaling, and in platelets it could therefore act as a downstream target of Rac1 in response to sCD40L. In fact, sCD40L induced a time-dependent activation of p38 MAPK, and inhibition of Rac1 with NSC23766 significantly reduced its activation, indicating that p38 MAPK does indeed act as a downstream target of Rac1 (Figure 5A). Furthermore, specific blockade of p38 MAPK with SB203580, which prevented its phosphorylation (Supplemental Figure II), impaired the effects of sCD40L on platelet P-selectin expression (Figure 5B) and aggregation (Figure 5C), thus highlighting its implication in these responses. These data establish Rac1 and its downstream effector p38 MAPK as key components involved in sCD40L signaling in platelets.

sCD40L Exacerbates Thrombus Formation and Leukocyte Infiltration

To date, no direct correlation between circulating levels of sCD40L and thrombosis has been established, and this could be of important clinical and physiopathological relevance. To explore this aspect, we injected sCD40L (0.25 mg/kg or approximately 5 μg/mouse) into WT and CD40−/− mice before vascular injury, thereby enhancing its plasma circulating levels to 47.5 ± 3.7 ng/mL (n = 4), and assessed thrombus formation. Infusion of sCD40L into WT mice significantly exacerbated thrombus formation, in comparison with vehicle-treated mice, in which occlusion was only partial (Figure 6). Interestingly, CD40−/− mice were protected from increased
levels of circulating sCD40L, as no significant difference in thrombosis between treated and nontreated groups was observed (Figure 6A and 6B), confirming the in vivo contribution of the CD40 receptor in sCD40L-induced thrombus formation. Because sCD40L induces surface expression of P-selectin on platelets (Figure 1B), we sought to determine the extent of leukocyte infiltration within the thrombus because of its involvement in platelet/leukocyte interactions, a well-established aspect of hemostasis known to potentiate thrombus formation.25 Leukocyte infiltration, as measured by CD45 immunostaining (Figure 6C) and optical quantification of histological sections postthrombosis (Figure 6D), was significantly increased in WT but not in CD40−/− mice that received sCD40L. These results establish a direct in vivo correlation between circulating levels of sCD40L and arterial thrombosis, while highlighting the requirement of the CD40 receptor in this process.

**Discussion**

CD40L has gained much attention over the years for its involvement in the pathogenesis of atherosclerosis, and today, numerous clinical studies show a tight association between levels of sCD40L and vascular diseases.1–4 The majority of sCD40L found in plasma is believed to originate from activated platelets, and this in turn has been shown to influence platelet activation. Here, we provide novel insights into the regulation of platelet function by CD40L, as we show that sCD40L primes platelets and enhances aggregation through a CD40-mediated TRAF-2/Rac1/p38 MAPK-dependent pathway. This ultimately leads to shape change and actin polymerization. Furthermore, we establish a direct correlation between circulating levels of sCD40L and thrombus formation.

To get insights into the cellular and molecular impact of sCD40L on platelet function, it was important that we first investigate its effect on platelet aggregation and activation. sCD40L was unable to trigger aggregation of resting platelets or to induce activation of the integrin αIIbβ3, whereas it significantly increased the expression of P-selectin, in accordance with previous studies.14,17,22 This may be attributed to the fact that sCD40L alone does not affect intraplatelet calcium...
Figure 4. The Rho-GTPase Rac1 is required for sCD40L signaling. A, VASP phosphorylation and Rac1 activation were detected from platelets left untreated (baseline) or incubated with sCD40L (1 μg/mL) for the indicated time. Control experiments were performed in parallel with 1 μg/mL of sCD40L<sup>RY</sup> and sCD40L<sup>WT</sup> after 30 minutes stimulation. Blots are representative of 4 independent experiments. B, Effect of Rac1 inhibition on sCD40L-induced potentiation of platelet aggregation. Platelets were preincubated with the Rac1 inhibitor NSC23766 (50 μmol/L) or vehicle dimethyl sulfoxide for 15 minutes at 37°C. Cells were then left unstimulated (control) or incubated with sCD40L (1 μg/mL). Aggregation was then monitored in the presence of a priming dose of collagen (0.25 μg/mL). Histogram represents the mean of data of aggregation traces (n=4; *P<0.05 versus sCD40L). C, Platelets were left untreated (baseline) or incubated with sCD40L (1 μg/mL) with or without NSC23766 (50 μmol/L) and assessed by flow cytometry for CD62P expression. Histogram shows the mean data of overlay plot (n=3; *P<0.05 versus sCD40L).
influx (Supplemental Figure III) or dense granule secretion, as assessed by ATP release and mepacrine uptake (Supplemental Figure IV), which are necessary for integrin activation and platelet aggregation. However, we found that sCD40L strongly enhanced platelet aggregation, P-selectin expression, and IIb/IIIa activation in response to subthreshold concentrations of platelet agonists, indicating that it rather acts as a broad and potent primer of platelets, such as matrix metalloproteinase-2, plasma protein growth arrest-specific 6, and stromal derived factor-1α.26–28 Moreover, this priming phenomenon holds true for B cell proliferation, as sCD40L requires costimulation with interleukin-4 for immunoglobulin E secretion.29,30 Taken together, this would indicate that CD40L acts as an accessory, but important, element in platelet function.

One important aspect of controversy regards the identification of the platelet receptor for sCD40L. In our study, we were able to show by molecular and genetic approaches that sCD40L enhances platelet function by interacting with CD40. These results are in agreement with previously published data showing that sCD40L can induce platelet activation and secretion of reactive oxygen species and the chemokine RANTES through binding to CD40.14,17,22 In addition to CD40, its constitutively expressed receptor on platelets, it has been reported that sCD40L binds to αIIBβ3 already in its active form), which is 40 times higher than the concentration used in the present study. This may suggest that CD40 and αIIBβ3 constitute the high-

Figure 5. The p38 MAPK is an important Rac1 downstream target in response to sCD40L. A, Platelets were left untreated (time 0) or stimulated with sCD40L (1 μg/mL), with or without NSC23766 (50 μmol/L), for the indicated time and assessed for p38 MAPK phosphorylation by SDS-PAGE. Results are expressed as fold increase in optical density (O.D.) over time 0 (n = 4; *P < 0.05). B, Platelets were left unstimulated (baseline) or incubated with sCD40L (1 μg/mL), with or without the p38 MAPK inhibitor SB203580 (5 μmol/L) and assessed for CD62P by flow cytometry. Histogram represents the mean of data of overlay plot (n = 5; *P < 0.05 versus sCD40L). C, Platelets were preincubated with SB203580 (5 μmol/L) or vehicle dimethyl sulfoxide for 15 minutes at 37°C. Cells were then left unstimulated (control) or incubated with sCD40L (1 μg/mL), and aggregation was then induced by a priming dose of collagen (0.25 μg/mL). Histogram represents the mean of data of aggregation traces (n = 4; *P < 0.05 versus sCD40L). p-p38 indicates phosphorylated p38.
and low-affinity receptors for sCD40L, respectively. However, additional studies are necessary to specifically address this issue. Thus, it appears that increased levels of circulating sCD40L, as seen in ACS patients, prime platelets via CD40, whereas its interaction with IIb/IIIa at the site of vascular injury, where higher levels of sCD40L may be generated, stabilizes platelet aggregates.

CD40 signaling in immune and endothelial cells requires its association to TRAF proteins. To our knowledge, the expression of TRAF members in platelets and their association with platelet CD40 following sCD40L stimulation are still unknown. Here, we found that only TRAF-2 associates with CD40 on ligation, indicating that it may be responsible for sCD40L/CD40-induced signaling in platelets. Although they are present in platelets, neither TRAF-1 nor TRAF-6 associate with CD40, indicating that they may instead play a role in tumor necrosis factor signaling. Even though CD40 contains binding sites for both TRAF-2 and TRAF-6, binding of either one is sufficient to induce activation of nuclear factor-κB in B cells. This would also hold true for platelets, as binding of TRAF-2 alone appears sufficient to trigger downstream signaling.

In platelets, Rac1 and VASP govern shape change, cytoskeletal reorganization, and spreading, through lamellipodia and filopodia formation, respectively. VASP is typically phosphorylated on 2 main residues, Ser239 and Ser157. Platelet inhibitors induce phosphorylation of both residues, whereas agonists that cause platelet activation trigger phosphorylation of VASP on Ser157, thereby promoting actin depolymerization and filopodia formation. Our finding that sCD40L promotes phosphorylation of VASP on Ser157 provides evidence for its role as an inducer of shape change in platelets. As further support for the role of sCD40L in these processes, we highlight its capacity to induce activation of the small GTPase Rac1. Rac1 activation in response to sCD40L was shown to be of physiological importance, given that its specific inhibition significantly reduced sCD40L’s ability to potentiate platelet aggregation and P-selectin expression. Interestingly, in accordance with our results, it has recently been shown that in endothelial and WEHI 231 B cells, CD40 signaling-induced reactive oxygen species generation requires activation of Rac1. Moreover, in immune cells, CD40L is classically known to induce activation of MAPKs, such as p38. Here, we were able to confirm that sCD40L induces activation of p38 MAPK in resting platelets, and further highlight its involvement in platelet activation and aggregation as a downstream effector of Rac1 in response to sCD40L. Consistent with its role in sCD40L-induced shape change and platelet priming, p38 MAPK has been shown to be an important regulator of actin polymerization and platelet spreading.

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. Here, we provide novel evidence demonstrating a direct correlation between enhanced levels of sCD40L and thrombosis. Mice that received sCD40L before vascular injury showed increased thrombus formation, indicating that they were predisposed to thrombotic stimulus. Our study adds new insights to a previous work showing that CD40L−/− mice develop unstable thrombi and that this deficiency can be overcome by infusion of 1.6 mg/kg sCD40L in a αIbβ3-dependent manner.15 Here, a different approach was used, as we increased circulating levels of sCD40L in mice by injecting 0.25 mg/kg to reach approximately 50 ng/mL of plasma sCD40L, to mimic conditions similar to those seen in patients with ACS. In this experimental setting, sCD40L was shown to exacerbate thrombosis in WT mice but not in CD40−/− mice, indicating that enhanced levels of sCD40L prime resting platelets in a CD40-dependent manner, predisposing them to enhanced thrombus formation. Because CD40−/− mice do not show a defect in thrombus formation, it is likely that CD40 is not essential for platelet hemostasis in the absence of significant levels of sCD40L but rather has a pathological importance in atherothrombosis in the presence of elevated levels of circulating sCD40L as seen in patients with ACS. Given that sCD40L induces platelet P-selectin expression, which is involved in platelet/leukocyte interactions14,17,20 and stabilization of thrombus mass,25 we measured the extent of leukocyte infiltration within the thrombus. We found significantly more leukocytes within the thrombus of sCD40L-treated mice, presumably accounting for the increase in thrombus formation seen in these animals, concomitantly with enhanced platelet predisposition to activation and aggregation in response to vascular injury.

Elevated levels of sCD40L are associated with increased cardiovascular risk, as seen in patients with ACS, such as unstable angina16 and acute myocardial infarction.1 However, the relative importance of the soluble form versus the membrane-bound form in thrombus formation is still unknown. It is likely that both forms are involved in primary hemostasis, whereas under pathological conditions, thrombosis is exacerbated by increased levels of the soluble form. Nevertheless, the involvement of CD40L in atherogenesis, thrombus formation, platelet-mediated inflammation, and plaque destabilization makes it a potential therapeutic target in atherothrombosis. Accordingly, it would be pertinent for future clinical studies to evaluate the degree of platelet priming in ACS patients, specifically through sCD40L-induced TRAF-2 association with CD40, for instance. Hence, a direct clinical link between sCD40L and platelet function could be established for pharmacological targeting.

In summary, aside from this newly identified TRAF-2/Rac1/p38 MAPK pathway involved in platelet priming in response to sCD40L, we highlight the relevance of sCD40L in a physiopathological setting of platelet function and thrombus formation. Indeed, enhanced levels of sCD40L potentiate platelet aggregation and exacerbate thrombus formation and leukocyte infiltration in response to vascular injury, in a CD40-dependent manner. This study provides novel evidence for the regulation of platelet function by sCD40L and may partly explain the link between levels of circulating sCD40L and the occurrence of cardiovascular complications. The CD40L/CD40 axis may ultimately represent a therapeutic target in the treatment of thromboinflammatory diseases.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents and antibodies

Recombinant human soluble CD40L (sCD40L) was obtained from R&D systems (Minneapolis, MN), while recombinant mouse soluble CD40L (msCD40L) came from Alexis Biochemicals (San Diego, CA). Recombinant human mutant sCD40L<sup>R/Y</sup> (Arginin (R) 203 for Alanin and Tyrosin (Y) 145 for Alanin) and its wild type counterpart sCD40L<sup>WT</sup> were generated as previously described. Antibodies against TRAF-1, -2, -3, and -6 were all purchased from Cell Signaling Technology (Beverly, MA). Anti-CD40 antibody used for immunoprecipitation of human CD40 was also from R&D systems, while anti-CD40 antibody used for detection of CD40 by immunoblotting came from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-VASP (Ser<sup>157</sup>), anti-phospho-p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>), anti-VASP (total) and anti-p38 (total), as well as the antibody against Rac1 were all procured from Cell Signaling Technology (Beverly, MA). Antibodies against P-selectin (AK4) and the active form of α<sub>IIb</sub>β<sub>3</sub> (PAC-1) were obtained from BD Biosciences (Mississauga, ON). The specific Rac1 NSC23766 inhibitor, the p38 SB203580 inhibitor and the actin polymerization Latrunculin B inhibitor were purchased from Calbiochem (San Diego, CA). Alexa Fluor 555-phalloidin came from Invitrogen (Carisbad, CA). Protein A agarose beads were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and p21-activated kinase-protein binding domain (PAK1-PBD) beads were from Cytoskeleton, Inc (Denver, CO). Native type I collagen and adenosine diphosphate (ADP) were from Chronolog Corp. (Havertown, PA), while human thrombin was purchased from Sigma-Aldrich (Oaskville, ON).
Animals
Age- and sex-matched wild type (WT) and CD40−/− mice, both on C57BLK/J6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under pathogen free conditions. Handling and care of animals were in compliance with guidelines established by the animal care and ethical committee of the Montreal Heart Institute.

Isolation of human and mouse platelets
Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment, in accordance with the guidelines of the human ethical committee of the Montreal Heart Institute.
Platelet-rich plasma (PRP) was obtained by centrifugation of acid citrate dextrose (ratio of 1:5) anticoagulated blood at 200g for 15 minutes. Platelets were then pelleted from PRP, to which 1 μg/mL of PGE₁ was added, washed with HBSS-Hank’s sodium citrate buffer (138 mM NaCl, 5 mM KCl, 0.34 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM Na₂HCO₃, 5.6 mM Glucose, 10 mM HEPES, 12.9 mM sodium citrate, pH 7.4), also containing PGE₁ (0.5 μg/mL), and finally resuspended in HBSS-Hank’s buffer containing 2 mM MgCl₂ and 2 mM CaCl₂.

Murine washed platelets were prepared from mice anesthetized with a mixture of 75 mg/kg of Ketamine (Vetalar, Belleville, QC) and 0.5 mg/kg of medetomidine (Domitor, Pfizer, Kirkland, QC). Blood was drawn by cardiac puncture in 1-cc syringes containing 50 μL of heparin (1000 iU/mL) and diluted (1:1) with modified Tyrode’s buffer (150 mM
NaCl, 2.5 mM KCl, 12 mM NaHCO₃, 2 mM MgCl₂, 2 mM CaCl₂, 1 mg/mL BSA, 1 mg/mL dextrose, pH 7.4), containing prostacyclin (0.2 μg/mL). PRP was obtained by centrifugation of blood at 164g for 8 minutes, to which prostacyclin (0.1 μg/mL) was added, and platelets were pelleted by centrifugation at 1000g for 5 minutes. Platelets were finally resuspended in modified Tyrode’s buffer.

Human and mouse platelets were adjusted to 250 x 10⁶ platelets/mL, unless otherwise specified, and allowed to rest at 37ºC for 30 minutes before further manipulation.

Flow cytometry analysis of platelet activation

Translocation of platelet P-selectin and activation of α₁bβ₃ were measured by flow cytometry, as previously described.² Platelets were preincubated with sCD40L prior to cell stimulation with agonists, fixed with 1% paraformaldehyde, washed and stained with saturating concentrations of anti-P-selectin antibody (AK4-PE conjugated) for 30 minutes or its isotype-matched control IgG. For measurement of α₁bβ₃ activation, PAC-1 antibody (FITC-conjugated) was incubated with platelet suspensions prior to activation with sCD40L and agonists. Samples were analyzed (20,000 events) on an Altra flow cytometer (Beckman Coulter, Mississauga, ON) and platelets were gated by their characteristic forward and side scatter properties.

Measurement of platelet aggregation

Aggregation of human and mouse washed platelets was monitored on a four-channel optical aggregometer (Chronolog Corp., Havertown, PA) under shear (1000 rpm) at 37ºC. sCD40L (human, mouse and the mutated R/Y and WT forms) was preincubated with
platelet suspensions under static conditions at 37°C, 30 minutes prior to addition of agonists, and traces were recorded until stabilization of platelet aggregation was reached.

**Immunoprecipitation of CD40**

Platelets (500 x 10^6/mL) were stimulated as indicated, pelleted, then lysed into ice-cold RIPA lysis buffer (1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM sodium-orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 2 μg/mL benzamidin) for 1 hour at 4°C. Lysates were sonicated on ice and pre-cleared with 100 μL of protein A agarose beads for 15 minutes at 4°C. Beads were then pelleted and the supernatant was incubated with 5 μg/mL of anti-CD40 antibody overnight at 4°C. Samples were treated with 100 μL of protein A agarose beads for 1 hour at 4°C and precipitated by centrifugation, washed three time with ice-cold RIPA lysis buffer, resuspended in 2X Laemmli buffer, and boiled for 5 minutes. Supernatants were analyzed by immunoblot for the presence of TRAF-1, -2, -3, and -6, as well as CD40.

**SDS-PAGE and immunoblotting**

Proteins were resolved in 8% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk for 1 hour, washed three times with TBS/T (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1 % Tween-20) and incubated with appropriate primary antibody overnight at 4°C. Following washing steps, membranes were labeled with horseradish peroxidase-conjugated secondary antibody for 1 hour,
washed and bound peroxidase activity was detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Waltham, MA).

**Rac1 activation assay**

Platelets (1 x 10^9/mL) were treated with sCD40L and reactions were terminated by addition of an equi-volume of ice-cold 2X lysis buffer (50 mM HEPES, 300 mM NaCl, 2% Igepal, 20% glycerol, 20 mM MgCl_2, 50 mM sodium fluoride, 2 mM EDTA, 2 mM sodium orthovanadate, 20 μg/mL leupeptin, and 20 μg/mL aprotinin). Lysates were then clarified by centrifugation at 14,000g for 5 minutes at 4ºC and supernatants were treated with 10 μg of PAK1-PBD beads for 1 hour at 4ºC. Beads were centrifuged and washed three times with ice-cold lysis buffer. They were then resuspended in 2X Laemmlli sample buffer, boiled for 5 minutes, and supernatants were analyzed by immunoblotting for the presence of Rac1.

GDP and GTPγS were used as negative and positive controls, respectively. Supernatants from lysates were treated with either GDP (1 mM) or GTPγS (100 μM) for 15 minutes at room temperature, and reactions were terminated by placing samples on ice and adding MgCl_2 (60 mM). Samples were then clarified by centrifugation, incubated with PAK1-PBD beads, and treated with the same procedure as described above.

**Scanning electron microscopy**

Platelets treated or not with sCD40L were allowed to immobilize on 2% bovine serum albumin (BSA)-treated glass coverslips for 30 minutes at 37ºC. Samples were then fixed in 2% paraformaldehyde overnight at 4ºC. Dehydration of surfaces was achieved by
placing samples in ethanol/water followed by amyl acetate/ethanol baths for 15 minutes each, increasing the ethanol/water proportion from 30% to 100% and the amyl acetate/ethanol proportion from 25% to 100%. Slides were subsequently coated with gold palladium particles and analyzed on a Hitachi S-4700 Field Emission Gun Scanning Electron Microscope (FEG-SEM).

**Actin polymerization assay**
Platelets treated or not with sCD40L in the presence or absence of Latrunculin B were allowed to immobilize on 2% BSA-treated glass coverslips for 30 minutes at 37°C. Samples were then fixed in 2% paraformaldehyde for 20 minutes at room temperature. Platelets were permeabilized with 0.05% Triton X-100 in 2% BSA and stained with Alexa555-phalloidin in 1% BSA for F-actin detection. Series of fluorescent confocal images (Z stacks) were acquired with a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany). Alexa555-phalloidin was visualized using a 543-nm helium-neon laser line and a 63x/1.4 plan-apochromat objective (Zeiss) was used for magnification (voxel size is 28 nm X 28 nm X 250 nm (X,Y,Z)). Final images were produced using the Zeiss LSM 510 software and saved as LSM files.

**Thrombosis model**
The effect of sCD40L on thrombus formation was determined in a FeCl₃ mouse carotid injury model, as previously described. Briefly, anesthetized C57BL6 WT and CD40⁻/⁻ mice were injected with sCD40L (0.25 mg/Kg) through the jugular vein, 5 minutes prior to FeCl₃ (4%) injury of the right carotid artery, and blood flow and time to thrombotic
occlusion (blood flow of 0 mL/minute) were measured with the aid of a miniature ultrasound flow probe (0.5 VB 552, Transonic Systems Ithaca, NY) interfaced with a flow meter (T206, Transonic Systems) and a computer-based data acquisition program (Iox 2.2.17.19, Emka, Falls Church, VA). Mouse plasmatic sCD40L was measured by ELISA (Bender MedSystems, San Diego, CA), according to the manufacturer’s instructions.

**Histology and Immunostaining**

Following *in vivo* thrombosis measurements in mice, injured and contralateral non-injured carotid arteries were excised, fixed in 10% buffered formalin and analyzed by hematoxylin and eosin staining or CD45 immunostaining for leukocytes infiltration within the thrombus mass, as previously described.\(^3\) Briefly, sections were embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin and eosin, or an anti-CD45 antibody (Santa Cruz, Santa Cruz, CA). Samples were visualized using an Olympus BX60 microscope (Olympus imaging America Inc, Center Valley, PA) and images were captured with a Retiga 2000R camera (QiImaging Corporation, Surrey, BC) and visualized through the Image Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD).

**Measurement of intracellular calcium flux**

Platelets in HBSS-Hank’s sodium citrate buffer were incubated with 5 μg/ml Fluo-4 AM for 30 minutes at 37°C. Platelets were then removed from excess Fluo-4 AM by centrifugation and resuspended in final HBSS-Hank’s buffer containing 2 mM MgCl\(_2\) and 2 mM CaCl\(_2\). Platelets were then placed onto a FluoroDish and mounted on a LSM
510 confocal microscope (Zeiss. Oberkochen, Germany). sCD40L (1 μg/ml) or thrombin (0.1 U/ml) was added to the platelet suspension and series of fluorescent confocal images were acquired in real-time at a rate of 30 images/second (excitation wavelength selected was 488 nm).

**Dense granule release**

ATP release was measured by a Lumi-Aggregometer according to the manufacturer’s instructions (Chrono-log Corp. Havertwon, PA). Briefly, 25 μl Luciferin-Luciferase (Chrono-Lume) reagent was added to a 475 μl platelet suspension 2 minutes before addition of sCD40L (1 μg/ml) or thrombin (0.1 U/ml).

Dense granule release was measured by mepacrine uptake into platelets. Platelets in HBSS-Hank’s sodium citrate buffer were incubated with 5 μM mepacrine (Quinacrine dihydrochloride, Sigma-Aldrich) for 30 minutes at 37°C. Platelets were then removed from excess mepacrine by centrifugation and resuspended in final HBSS-Hank’s buffer containing 2 mM MgCl₂ and 2 mM CaCl₂. Secretion of dense body constituents was evaluated by flow cytometry as the fluorescence remaining in platelets upon stimulation with sCD40L or thrombin; in comparison to resting platelets.

**Statistical analysis**

Results are presented as mean ± SEM of at least 3 independent experiments. Statistical comparisons were done using a one-way ANOVA, followed by a Dunnetts-t-test for comparison against a single group. Data with p<0.05 were considered statistically significant.
Supplemental References


Supplemental Figure I: Specific binding of CD40 ligand to CD40- or αIIbβ3-coated well plates. Plates were coated with 4 μg of soluble hCD40 or αIIbβ3 overnight at room temperature. After extensive washing, plates were blocked with 1% BSA, then recombinant soluble hsCD40L wild type (WT) or hsCD40L R/Y was added overnight at a concentration of 100 ng/mL. A polyclonal anti-hCD40L-biotinylated antibody was used followed by addition of striptavidin-HRP. The signal was detected by addition of TMB substrate. The reaction was halted by the addition of 1N H₂SO₄. Optical density (O.D.) was then measured by spectrophotometry.
Supplemental Figure II: Effect of SB203580 (0.1 μM -10 μM) on sCD40L-induced p38 MAPK phosphorylation. Resting platelets were preincubated with the indicated concentration of SB203580 for 15 minutes at 37°C or left untreated, and then stimulated with sCD40L (1 μg/mL). Total platelet lysates were analyzed by SDS-PAGE for phospho-p38 MAPK. Blot shown is representative of 3 independent experiments.
Supplemental Figure III: Effect of sCD40L on intracellular calcium flux. Intracellular calcium was measured by Fluo-4 AM fluorescence by real-time confocal microscopy following stimulation of platelets with either sCD40L (1 μg/mL) or thrombin (0.5 U/mL), as described in materials and methods. Tracings are representative of 3 experiments.
Supplemental Figure IV: Effect of sCD40L on platelet dense granule secretion. A, ATP secretion as measured by Luciferase assay (Chrono-Lume, Chrono-log). Results are expressed as a measure of increase in luminescence. Blot is representative of 3 independent experiments. B, Dense granule secretion was evaluated by measuring the loss of mepacrine fluorescence following activation by sCD40L (1 µg/mL) or thrombin (0.5 U/mL); in comparison to untreated cells which was set as 100% fluorescence (n=3; *P< 0.05 vs. baseline).
Supplemental Figure V: Effect of sCD40L on high dose collagen. Washed platelets were incubated with sCD40L (1 μg/mL) or left untreated for 30 minutes at 37 C, then stimulated with collagen (aggregation: 2 μg/mL; flow cytometry: 5 μg/mL), and assessed by optical aggregometry and flow cytometry for CD62P expression and α_{IIb}β_{3} activation, as described in materials and methods.