CD40 Ligand Influences Platelet Release of Reactive Oxygen Intermediates

Subrata Chakrabarti, Sonia Varghese, Olga Vitseva, Kahraman Tanriverdi, Jane E. Freedman

Objective—Soluble CD40 ligand (sCD40L) has been recently implicated in the pathogenesis of atherosclerosis. Elevated levels of sCD40L in acute coronary syndrome patients suggests enhanced platelet function; however, the exact mechanism by which this occurs is unknown. In this study, we examined the effect of sCD40L on platelet function and reactive oxygen and nitrogen species (RONS) generation.

Methods and Results—Platelet stimulation in the presence of recombinant sCD40L (rsCD40L) led to enhanced generation of RONS as measured by DCFHDA oxidation and confocal microscopy. Incubation with rsCD40L led to enhanced platelet P-selectin expression, aggregation, and platelet-leukocyte conjugation. Platelets isolated from CD40L-deficient mice had decreased agonist-induced NO release as compared with wild-type mice. Incubation of platelets with rsCD40L enhanced stimulation-induced p38 MAP kinase and Akt phosphorylation.

Conclusion—Soluble CD40L enhances platelet activation, aggregation, and platelet-leukocyte conjugation, as well as increases stimulation-induced platelet release of RONS through activation of Akt and p38 MAP kinase signaling pathways. These data suggest that sCD40L regulates platelet-dependent inflammatory and thrombotic responses that contribute to the pathogenesis of atherothrombosis. (Arterioscler Thromb Vasc Biol. 2005;25:2428-2434.)

Key Words: platelets ■ reactive oxygen species ■ CD40 ligand ■ fluorescence

CD40 ligand (CD40L, CD154) is a transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily. Originally identified as a surface molecule expressed on activated T-cells, CD40L was shown to be important for the induction of B-cell development and proliferation, as well as for the IgM to IgG isotype switch.1-3 CD40L is expressed in a variety of other cell types including monocytes, macrophages, dendritic cells, mast cells, basophils, eosinophils, B-cells, and platelets.3-6 CD40L is involved in the initiation of an inflammatory response at the vessel wall by inducing the expression of adhesion molecules and the secretion of chemokines by vascular endothelial cells.7

In addition to the trimeric membrane-bound form, CD40L also exists in a soluble 18-kDa form (sCD40L) that is released from platelets after stimulation.5,6,9 In contrast to membrane-bound CD40L, sCD40L fails to induce an inflammatory response in endothelial cells.5 Soluble CD40L has been shown to stabilize arterial thrombii10 and is reportedly a platelet GPIIIb/IIa ligand.11 In addition, sCD40L participates in platelet outside-in signaling after binding via its KGD motif.12

Platelets are the richest source of sCD40L in the circulation,13,14 and platelet aggregation and activation is associated with the release of sCD40L.7 A recent study suggests that platelet gp91phox contributes to the regulation of the release of CD40 ligand from platelets.15 Platelet activation and aggregation also induce oxidative stress16,17 through the generation of reactive oxygen and nitrogen species (RONS)18-20; however, the role of CD40L in the generation of RONS by platelets is unknown. In the current study, using CD40L-deficient mice and recombinant CD40 ligand (rsCD40L), we examined the effect of sCD40L on RONS regulation in platelets. The role of the stress response protein kinases Akt and p38 MAP kinase, known to mediate RONS generation, was also examined.

Materials and Methods
For detailed Materials and Methods, please see the data supplement available online at http://atvb.ahajournals.org.

Results
Prolonged Release of sCD40L After Platelet Stimulation
Platelets have recently been identified as a source of inflammatory chemokines and cytokines.8,21,22 As shown in Figure 1, platelet activation by thrombin results in the release of sCD40L as a function of time, whereas unstimulated platelets release only minor amounts of sCD40L. Recent data suggesting a role for sCD40L in platelet activation23 have important implications in atherosclerosis as unrestricted release of sCD40L by activated platelets may serve as a positive
feedback mechanism, amplifying activation of other platelets.\textsuperscript{24}

### The Functional Effects of sCD40L

To verify the functional effects of sCD40L, platelet aggregation and P-selectin expression by using flow cytometry were measured after incubation with rsCD40L. As shown in the Table, rsCD40L enhances platelet P-selectin expression even at ng/mL concentration. In addition, rsCD40L significantly enhances platelet aggregation. Because P-selectin expression may lead to platelet–leukocyte interaction, the effects of rsCD40L on platelet–neutrophil and monocyte conjugate formation in human blood were studied. As shown in Table I (available online at http://atvb.ahajournals.org), rsCD40L enhances ADP induced platelet–neutrophil and monocyte conjugate formation. In addition, rsCD40L alone induces platelet–monocyte conjugation.

### Recombinant Soluble CD40L Induces Platelet Release of Reactive Oxygen Intermediates

Although the role of sCD40L in the generation of ROS has been suggested in other settings,\textsuperscript{25,26} the role of sCD40L in the modulation of RONS generation in platelets is unknown. Figure 2A depicts levels of platelet-derived RONS as measured by oxidation of the redox sensitive probe, DCFHDA. The addition of rsCD40L significantly enhances generation of RONS in the TRAP-stimulated platelets in a dose-dependent fashion (Figure 2B).

Recently, it has been shown that CD40L is a glycoprotein IIb/IIIa ligand that induces platelet outside-in signaling.\textsuperscript{12} To determine the receptor specificity for CD40L-dependent generation of RONS, the effect of CD40L antibody or GPIIb/IIIa inhibition on the rsCD40L-induced platelet generation of RONS was studied. As shown in Figure 2C, both anti-CD40 ligand antibody (10 \( \mu \)g/mL, 95% blockage) or GPIIb/IIIa blocking antibody (abciximab; 20 \( \mu \)g/mL, 93% blockage) on rsCD40L-induced DCFHDA oxidation in the setting of TRAP stimulation (n=3; *P<0.05).

### Table I

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>P-Selectin Positive Platelets (Fold Change)</th>
<th>Aggregation (Fold Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10 ng/mL</td>
<td>2.44±0.395 *</td>
<td>3.7±1.5 **</td>
</tr>
<tr>
<td>100 ng/mL</td>
<td>4.01±1.41 *</td>
<td>4.9±1.2 **</td>
</tr>
</tbody>
</table>

*P<0.02; **P<0.01.
platelets. To define the specific source of RONS, platelets were incubated with the NOS inhibitor, L-NAME, or the NADPH oxidase inhibitor, apocynin. Both L-NAME and apocynin inhibited rsCD40L-induced RONS generation (Figure I, available online at http://atvb.ahajournals.org) suggesting that both eNOS and NADPH oxidase contribute to the observed DCFHDA oxidation. Additionally, rsCD40L induces platelet superoxide in a dose dependent manner (Figure I) even at low concentrations (10 ng/mL to 1 μg/mL).

The role of rsCD40L in platelet nitric oxide generation

To determine whether CD40L deficiency alters platelet-derived NO release, platelets were isolated from CD40L-deficient mice and stimulation-induced (thrombin, ADP, or collagen) NO generation was determined. As shown in Figure 4A, CD40L deficiency significantly impairs platelet release of NO. Notably, this attenuation in NO generation was apparent in response to all 3 agonists examined. We confirmed these results by carrying out platelet aggregation, with normal human platelets, in the presence of rsCD40L. The presence of varying concentration of rsCD40L enhanced platelet NO generation (Figure 4B). The rsCD40L-induced NO generation was attenuated by anti-CD40L antibody (Figure II, available online at http://atvb.ahajournals.org) indicating specificity of the rsCD40L induced effect.

The Effect of rsCD40L on Phosphorylation of Akt and p38 MAP Kinase

**The Effect of rsCD40L on Phosphorylation of Akt**

Recent studies have identified Akt as an important stress-response protein kinase in endothelial cells responding to changes in the redox environment. The observation that rsCD40L enhances oxidative stress in platelets led to the examination of the status of Akt phosphorylation in the presence and absence of platelet stimulation. As shown in Figure 5A, addition of rsCD40L clearly enhances Akt phosphorylation in thrombin- and TRAP-stimulated platelets. Moreover, this enhanced Akt activation is reversed in the presence of the PI3 kinase inhibitor, LY294002 (ELISA results subsequently confirmed by Western blot analysis; Figure III, available online at http://atvb.ahajournals.org). Interestingly, the addition of rsCD40L alone significantly increases Akt phosphorylation. These data demonstrate the ability of rsCD40L to induce Akt activation in unstimulated and stimulated platelets.
The Effect of rsCD40L on p38 MAP Kinase Phosphorylation

p38 MAP kinase has also been reported to regulate the stress–response mechanisms of vascular cells such as endothelial cells, smooth muscle cells, and human macrophages. In addition, a recent study showed that rsCD40L is capable of inducing platelet p38 MAP kinase phosphorylation. Therefore, we examined the role of p38 MAP kinase activation under conditions where rsCD40L induces platelet RONS generation. Platelets were stimulated with thrombin or TRAP, and p38 MAP kinase phosphorylation was determined. As shown in Figure 5B, like Akt, p38 MAP kinase phosphorylation was significantly enhanced in the presence of rsCD40L after thrombin stimulation. The phospho-ELISA plots shown in Figure 5B (confirmed also by Western blot; Figure IV, available online at http://atvb.ahajournals.org) indicate that the addition of rsCD40L alone, or in combination with TRAP, significantly enhances p38 MAP kinase phosphorylation. Phosphorylation of p38 MAP kinase was attenuated in the presence of specific MAP kinase inhibitor, SB203580 (Figure 5B and Figure IV).

Involvement of PI3 Kinase/Akt and p38 MAP Kinase in Platelet RONS Generation

To understand the role of PI3 kinase/Akt or p38 MAP kinase in rsCD40L-induced platelet RONS generation, we carried out flow cytometric analysis monitoring DCFHDA oxidation (as described in Figure 2A) in presence of specific kinase inhibitors. Presence of the PI3 kinase inhibitor, Ly294002, and the p38 MAP kinase inhibitor, SB203580, eliminated platelet activation–induced RONS generation (Figures V and VI, available online at http://atvb.ahajournals.org).

Discussion

During inflammation, reactive oxygen species promote the adhesion of blood cells to the vascular endothelium by eliciting production of inflammatory mediators or activating nuclear transcription factors that bind to genes encoding adhesion molecules and cytokines. CD40–40L signaling represents another pathway that enables blood cells to amplify the endothelial cell responses to inflammation and contribute to the regulation of hemostasis. Platelets can indirectly orchestrate (through CD40–40L interaction via the endothelium) changes in coagulation, leukocyte trafficking, and extracellular matrix modeling turnover. CD40L has been associated also with numerous vascular diseases including diabetes and acute coronary syndromes. In recent studies, the prognostic values of serum sCD40L in acute coronary syndromes and in unstable angina patients has been reported.

Reports of sCD40L release by platelets have led to studies examining the mechanism of CD40L expression, release, and its regulatory role in the inflammatory milieu of the vasculature. Platelet activation is accompanied by release of both sCD40L and RONS such as superoxide and NO; however, the role of platelet-derived sCD40L in the generation of RONS is unknown. In the present study, we examined the effect of CD40L on the generation of RONS in platelets and determined the mechanism for the observed effects. Initially, we demonstrated a prolonged release of...

Figure 5. The effect of rsCD40L on platelet AKT phosphorylation (A) or p38 MAP kinase phosphorylation (B). i, The effect of rsCD40L on thrombin-induced AKT/p38 MAP kinase phosphorylation. Platelet protein phosphorylation was determined after 5 minutes preincubation at 37°C with rsCD40L (10 μg/mL) followed by thrombin (1 u/mL) stimulation. ii, Quantitative evaluation (ELISA) of the effect of rsCD40L on AKT/p38 MAP kinase phosphorylation after TRAP stimulation (50 μmol/L). Platelets were preincubated with rsCD40L (10 μg/mL) at 37°C for 5 minutes followed by TRAP stimulation (n=4; *P<0.05 compared with resting platelets; **P<0.002 compared with TRAP stimulation [Akt]; ***P<0.002 compared with rsCD40L+TRAP stimulation [Akt]; ****P<0.02 compared with TRAP stimulation [p38 MAP kinase]; +P<0.02 compared with rsCD40L+TRAP stimulation [p38 MAP kinase]). [LY29004]=25 μmol/L. [SB203580]=10 μmol/L.
sCD40L by platelets for 6 hours after thrombin stimulation (Figure 1). Sustained release of platelet sCD40L might contribute to atherothrombotic disease \cite{13,50} by forming platelet–platelet (CD40–40L\textsuperscript{51}/CD40L–GPIIb/IIa),\textsuperscript{10} platelet–leukocyte (CD40L–40P-selectin–PSGL),\textsuperscript{52} and platelet–endothelial (CD40L–CD40) conjugates.\textsuperscript{26} As shown in the Table and Table I, rsCD40L is capable of inducing platelet P-selectin expression and platelet–leukocyte conjugate formation in whole blood. Recombinant CD40L has also been recently reported to activate platelets,\textsuperscript{23,51} resulting in secretion in vivo.

P-selectin expression and platelet–leukocyte conjugate formation in whole blood. Recombinant CD40L has also been recently reported to activate platelets.\textsuperscript{23,51} resulting in secretion of P-selectin and suggesting the possibility of in vivo platelet-induced inflammation. Recent work by Wagner et al\textsuperscript{53} has provided direct experimental evidence that CD154 (CD40L) can induce the expression of CD154 in endothelial cells and influence atherogenesis through activation of extravasating monocytes. Additionally, CD40L positive platelets have been shown to induce CD40L expression de novo in endothelial cells.\textsuperscript{54} These observations support the proposed coordinated interactions among platelets and leukocytes with activated endothelium in diseased blood vessels through adhesion molecules and, particularly, CD40/40L.\textsuperscript{55}

Relevant to vascular inflammation is the effect of CD40L on RONS generation. Figures 2 and 3 show that rsCD40L increases stimulation-induced RONS generation by platelets and that this effect is reversible with specific antibodies. To determine whether platelet RONS generation is thrombin receptor–specific, we carried out further flow cytometric analysis with other agonists. In contrast to TRAP stimulation, we observed minimal generation of reactive oxygen species after ADP stimulation (data not shown). It is possible that the TRAP-induced RONS generation may be thrombin receptor–specific and independent of purinergic stimulation. Inhibition of the GPIIb/IIIa complex also decreased RONS generation, consistent with the previous observation showing that sCD40L is a GPIIb/IIIa ligand.\textsuperscript{10} Mechanistically, rsCD40L may ligate the platelet receptor CD40 or GPIIb/IIIa complex to initiate platelet signaling pathways that affect the generation of reactive oxygen and nitrogen species. In a clinical setting, recent studies have also shown that GPIIb/IIIa antagonists reduce circulating sCD40L and leukocyte–platelet aggregate formation in patients with acute coronary syndromes undergoing percutaneous coronary intervention.\textsuperscript{26}

The importance of CD40L in platelet generation of RONS was shown using CD40L-deficient mice (Figure 4A). The finding of impaired NO generation was complemented by the studies in human platelets where NO release was enhanced on addition of rsCD40L (Figure 4B). Additionally, CD40L-deficient mice had a lower extent of platelet DCFHDA oxidation as compared with wild-type mice platelets after thrombin stimulation (data not shown). It is possible that the combined production of NO mediated by Akt and/or p38 MAP kinase and superoxide mediated by p38 MAP kinase activation leads to the generation of peroxynitrite/RONS causing rsCD40L/TRAP-induced DCFHDA oxidation and fluorescence induction. The formation of peroxynitrite is further suggested by platelet fluorescence inhibition studies using NOS inhibitor, l-NAME, and NAPD oxidase inhibitor, apocynin (Figure 1). p38 MAP kinase may also stimulate the PI3 kinase/Akt/eNOS pathway, enhancing NO generation.\textsuperscript{57} TRAP-induced platelet DCFHDA oxidation study (Figures V and VI) indicates that both PI3 Kinase/Akt and p38 MAP kinase signaling pathways are involved in the rsCD40L-dependent RONS generation.

Some earlier studies have also documented that platelet generation of reactive oxygen intermediates\textsuperscript{20} involves PI3 kinase/Akt and MAP kinase activation. To investigate whether rsCD40L-induced fluorescence is accompanied by Akt and p38 MAP kinase activation, we analyzed platelet proteins after stimulation in the presence or absence of rsCD40L. The results (Figure 5A) indicate that rsCD40L enhances Akt phosphorylation in both stimulated and unstimulated platelets. Akt is an endothelial cell stress–response protein\textsuperscript{27,28} that counteracts external oxidative stress by enhancing NO generation after eNOS phosphorylation.\textsuperscript{28,58}

Enhanced Akt phosphorylation is also consistent with increased NO generation as observed during platelet aggregation (Figure 4B). These results suggest that, similar to endothelial cells,\textsuperscript{28} rsCD40L may regulate pathways leading to enhanced NO formation.

In vascular cells, p38 MAP kinase is also known to regulate RONS-dependent pathways.\textsuperscript{29,30,59,60} Similar to Akt, rsCD40L also induced p38 MAP kinase phosphorylation (Figure 5B). This observation is consistent with a recent report showing increased platelet p38 MAP kinase phosphorylation in response to rsCD40L.\textsuperscript{23} MAP kinase may enhance the generation of superoxide by stimulating NADPH oxidase as inhibition of NADPH oxidase with apocynin\textsuperscript{61} decreased platelet generation of RONS (Figure 1). We also examined whether involvement of p38 MAP kinase signaling is linked with platelet calcium mobilization during TRAP-induced fluorescence induction. Our findings (Figure VII, available online at http://atvb.ahajournals.org) suggest that p38 MAP kinase inhibition does not alter TRAP/rsCD40L-induced platelet calcium mobilization. We have also verified that rsCD40L/TRAP-induced platelet RONS generation is sensitive to calcium inhibition by dimethylbapta (data not shown).

In our findings there appears to be a discrepancy between the degree of enhancement of rsCD40L-induced Akt and p38 MAP kinase phosphorylation as compared with fluorescence induction (Figures 5 and 2A). There are several possible explanations for this difference, including (1) the use of distinct experimental conditions, eg, temperature variations: whereas we carried out the TRAP-induced fluorescence induction study at room temperature, TRAP-stimulated phosphorylation studies were done at 37°C; (2) Measurement procedures: in flow cytometry we directly measure the fluorescence induction, whereas protein phosphorylation evaluation involves a multi step procedures. An approach involving simultaneous measurement of fluorescence induction and protein phosphorylation can only provide the exact stoichiometry for these 2 experiments.

There are some limitations to these studies. We carried out platelet stimulation–induced sCD40L release in washed platelets. However, in physiological conditions, platelet stimulation– and activation-induced sCD40L release may be confounded by numerous plasma proteins. Interestingly, recent studies\textsuperscript{43} with platelet rich plasma reported iso-TRAP stimulation–induced sCD40L release in the range of 0.33 to
Acknowledgments

We thank Dr. Michael Kirber for his help and encouragement during confocal microscopic studies.

References


CD40 Ligand Influences Platelet Release of Reactive Oxygen Intermediates
Subrata Chakrabarti, Sonia Varghese, Olga Vitseva, Kahraman Tanriverdi and Jane E. Freedman

Arterioscler Thromb Vasc Biol. 2005;25:2428-2434; originally published online September 1, 2005;
doi: 10.1161/01.ATV.0000184765.59207.f3
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/25/11/2428

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/09/01/01.ATV.0000184765.59207.f3.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for
which permission is being requested is located, click Request Permissions in the middle column of the Web
page under Services. Further information about this process is available in the Permissions and Rights
Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org//subscriptions/
CD40 Ligand Influences Platelet Release of Reactive Oxygen Intermediates

Subrata Chakrabarti, Sonia Varghese, Olga Vitseva,
Kahraman Tanriverdi, and Jane E. Freedman

Materials and Methods

Recombinant CD40 ligand (rsCD40L) was purchased from R & D systems (Minneapolis, MN). Dichlorodihydrofluorescein diacetate (DCFHDA) and MitoTracker Red CM H2XROS, Fluo3-AM and Dimethylbapta-AM were purchased from Molecular Probes (Eugene, OR). Apocynin (Acetovanillone) was purchased from Aldrich Chemical Company (Milwaukee, WI). Thrombin receptor activating peptide (TRAP) was purchased from Peninsula Laboratories (San Carlos, CA). Dihydrorhodamine was purchased from Cayman Chemicals (Ann Arbor, MI). Human alpha thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). Anti-p38 MAP kinase antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-p38 MAP kinase (Thr180/Tyr180), anti-Akt and anti-phospho-Akt (ser473/472) antibodies were purchased from BD Biosciences (San Jose, CA). HRP-conjugated anti-mouse antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-rabbit IgG-HRP antibodies were procured from Cell Signaling Technology (Beverley, MA). ELISA kits for
phospho-p38 MAP Kinase and phospho-Akt were obtained from Biosource (Camarillo, CA). Blocking anti-CD40 ligand antibody was purchased from Coulter (Immunotech, Fullerton, CA). Total amounts of sCD40L released by activated platelets were measured by ELISA (Bender Med Systems, Burlingame, CA). Anti-β-actin monoclonal antibody was purchased from Novus Biologicals (Littleton, CO). PE-labeled anti P-selectin antibody (CD62P-PE), CD14-FITC and CD41-PE-Cy5 was obtained from Pharmingen-BD Biosciences (San Diego, CA). Homozygous CD40L (-/-) and C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Platelet activation and release of soluble CD40 ligand

Peripheral blood was drawn from healthy adult volunteers who had not consumed acetylsalicylic acid or any other platelet inhibitor for at least seven days. The protocol was approved by the Boston Medical Center Ethics Committee on Human Research.

Briefly, blood was drawn into a 10% sodium citrate containing syringe using a 21-gauge needle. The citrated blood was centrifuged (150 g, 15 min, 22°C) and the supernatant (PRP) was separated. Platelet pellets were obtained by a second centrifugation (392 g, 20 min, 22°C) of the PRP after mixing with equal volume of platelet wash buffer (10mM sodium citrate, 150mM NaCl, 1mM EDTA, 1% dextrose, pH 7.4) supplemented with 0.2µM prostaglandin E₁. Platelet pellets were briefly washed twice with HEPES buffer (140mM NaCl, 6mM KCl, 2mM MgSO₄, 7H₂O, 2mM Na₂HPO₄, 6mM HEPES, pH 7.4) before its final
resuspension in the same buffer. Resuspended platelets were kept in room temperature and utilized within 2 hours of isolation.

Platelets were treated with 0.2 u/ml thrombin to induce platelet activation, and the amount of sCD40L released from thrombin-activated platelets was determined as a function of time. Samples were collected by (micro) centrifugation at 11000g at 4ºC for 5 minutes after the specified time of incubation. The supernatant was stored at -20ºC prior to measurement of sCD40L levels by ELISA. The sCD40L levels from the platelet supernatant were measured by a commercially supplied kit (High Sensitivity ELISA kit, Bender Med Systems). Briefly, 20µl samples (mixed with 80µl standard diluent buffer) and 100µl standards in duplicate were loaded in the ELISA plate wells and processed according to manufacturer's instruction. The color developed was measured at 450nm using 650nm as the reference wavelength. The concentration of the sCD40L released from the samples was determined from the linear portion of the standard curve.

Platelet P-selectin Expression, Aggregation and Platelet—Leukocyte Conjugate Formation

To study the functional effect of sCD40L on platelets, activation induced expression of P-selectin and platelet aggregation were examined. By flow cytometry, we also verified the effect of rsCD40L on platelet-leukocyte conjugate formation in whole blood.
**P-selectin expression study**

Washed platelets in presence and absence of recombinant CD40L (rsCD40L) were incubated in HEPES buffer for 8 minutes at 37°C. For comparison purposes, selected samples were also stimulated with 20µM TRAP. Samples were fixed with 1% fresh formaldehyde for 10 minutes at room temperature. Platelets were collected by centrifugation (2700 g, 1 min), resuspended in PBSG (0.5 % BSA and 5.5 mM D-glucose in PBS) and then incubated (20 min, 22°C, in dark) with PE-labeled monoclonal CD62P antibody or corresponding PE-labeled isotype controls. Platelet samples were washed and resuspended in PBSG and analyzed in a FACScan flow cytometer (Becton Dickinson) or in a Fluorescence Activated Cell Sorter (Dako Cytomation). At least ten thousand platelet-specific events were collected for each set of data points. Flow cytometric data were analyzed by histogram statistics (CellQuest software, Becton Dickinson or Summit V3.1 Build 844).

**Platelet Aggregation**

Washed platelets were incubated in presence and absence of rsCD40L for 5 minutes at 37°C. Platelets supplemented with fibrinogen were subjected to aggregation in a four channel Platelet Aggregation Profiler (Biodata Corporation). Thrombin receptor activation peptide was used as an initiator of aggregation after a stable baseline was obtained.
Analysis of Platelet-Leukocyte Conjugates

Human blood was incubated with rsCD40L (10µg/ml) in presence and absence of ADP (4µM) for 15 minutes at room temperature. Platelet-leukocyte conjugates were analyzed by using two-color flow cytometry in a similar manner as described earlier\(^2\) where CD14-FITC was used as a monocyte marker and CD41-PE-Cy5 as a marker for platelets. EDTA (10mM) was used as a negative control for conjugate formation.

Induction of platelet aggregation and measurement of nitric oxide

A NO selective microelectrode was used to monitor platelet aggregation-induced NO production as described previously.\(^3,4\) Briefly, platelets (2x10\(^8\)/ml) were washed and incubated with rsCD40L or vehicle control for 5 minutes at room temperature. Aggregation was induced by appropriate agonists as described previously.\(^3\)

Assessment of reactive oxygen species by flow cytometry

Freshly isolated, washed platelets were incubated with the fluorescent probe, DCFHDA, or vehicle control for 5 minutes. Samples were then stimulated for 3 minutes with TRAP (20µM) and immediately analyzed using a Fluorescence Activated Cell Sorter (Dako Cytomation). At least ten thousand platelet-specific events were collected for each set of data points. The TRAP-induced mean fluorescence induction was calculated after gating the stimulated platelet
population. Unstimulated platelets not receiving any fluorophore were used as a negative control.

**Measurement of Platelet Aggregation induced Superoxide Release**

Platelet aggregation induced superoxide generation was determined as described earlier. Briefly, washed platelets were incubated with varying concentration of rsCD40L or control buffer for 5 minutes at 37°C. Superoxide released following PMA (150nM) induced platelet aggregation was measured in a lumiaggregometer using lucigenin as a luminescent agent.

**Assessment of reactive oxygen species and calcium mobilization by confocal microscopy**

Fluorescence confocal images were obtained using a two-photon laser-scanning microscope. Platelets, at a concentration of 2x10^8 /ml, were incubated for 10 minutes with 20µM dihydrorhodamine or 0.5µM of the redox sensitive MitoTracker Red probe. A baseline reading was obtained prior to platelet activation. Images were recorded at different time points following stimulation, and the fluorescence data was analyzed using NIH image software after background subtraction.

Platelet calcium mobilization was measured using calcium sensitive probe Fluo3-AM. Washed platelets in HEPES buffer were incubated with 2.5µM Fluo3-AM for 45 minutes at room temperature. In order to verify the specificity of the calcium response, some samples were treated with the calcium chelator Dimethylbapta-
AM (Molecular Probes). For other studies, specific samples were treated for 10 minutes with rsCD40L and/or p38 MAP kinase inhibitor SB203580 (10µM). Following the capture of baseline fluorescence, samples were stimulated with TRAP (50µM) and fluorescence induction was continuously monitored by confocal microscopy. Data were analyzed using NIH image software after background subtraction.

**Analysis of proteins following platelet stimulation**

Washed platelets (2x10^8 /ml) were pre-incubated at 37°C in presence or absence of rsCD40L for 5 minutes and then stimulated with thrombin for 3 minutes. The reaction was terminated by the addition of 3XSDS sample buffer. Protein samples were subjected to western blot analysis using standard procedures. Protein blots were probed with anti-phospho-Akt (1:1000) and anti-phospho-p38 MAP kinase (1:1000) antibodies in TBST containing 1% BSA. Total Akt (1:500) and p38 MAP kinase (1:2500) or β-actin (1:5000) was probed in TBST supplemented with 5% milk. The blots were developed with HRP-conjugated anti-mouse (1:4000) or anti-rabbit (1:10000) secondary antibodies.

In other experiments, a commercially available ELISA kit (Biosource) was employed to quantify Akt and p38MAP Kinase phosphorylation. Briefly, washed platelets (7.5 x10^8 /ml) were pre-incubated at 37°C with vehicle control, PI3 kinase inhibitor (LY29004, 25µM), or p38 MAP kinase inhibitor (SB203580, 10µM) for 5 minutes in presence or absence of rsCD40L. Afterwards, platelets were stimulated with TRAP (50µM) for an additional 3 minutes at 37°C. The
reaction was terminated by the addition of lysis buffer (Biosource) supplemented with protease and phosphatase inhibitors (Sigma). The samples were lysed on ice for 30 minutes with occasional stirring and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was stored in aliquots at -70°C.

**Statistical analysis**

Data are expressed as mean ± standard deviation. Analysis of differences between group means was evaluated with one-way analysis of variance (ANOVA). P<0.05 was accepted as statistically significant.

**References**

Table I: Recombinant soluble CD40L (rsCD40L) enhances platelet-leukocyte conjugate formation in human blood.

<table>
<thead>
<tr>
<th>Platelet-leukocyte conjugates</th>
<th>None</th>
<th>EDTA</th>
<th>rsCD40L</th>
<th>ADP</th>
<th>ADP + rsCD40L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-monocyte (%)</td>
<td>19.64 ± 0.53</td>
<td>5.46 ± 1.23</td>
<td>27.4 ± 3 *</td>
<td>56.43 ± 5.6</td>
<td>80 ± 7 **</td>
</tr>
<tr>
<td>Platelet-neutrophil (%)</td>
<td>6.93 ± 1.48</td>
<td>5.93 ± 1.4</td>
<td>8 ± 1</td>
<td>17 ± 2</td>
<td>51 ± 9 **</td>
</tr>
</tbody>
</table>

n=3, *P<0.05 vs unstimulated, **P<0.01 vs ADP. [rsCD40L] =10µg/ml; [ADP] =4µM, EDTA (negative control) =10mM
Figure I: (A) TRAP and rsCD40L (10ng/ml) induced platelet DCFHDA oxidation in the presence of a NOS inhibitor (L-NAME; 300µM) or NADPH oxidase inhibitor (apocynin; 200µM) (n=3, *P<0.05) (B) The effect of low concentrations of rsCD40L (10ng/ml to 1µg/ml) on platelet aggregation induced (150nM PMA) superoxide generation as measured by lucigenin chemiluminescence (n=3, *P<0.02).
Figure II: Washed human platelets were pre-incubated with 1.8µg/ml rsCD40L in presence and absence of 3.6µg/ml anti-CD40 ligand antibody for 5 minutes and then TRAP aggregation induced NO generation was measured. 

(n=3; *P<0.01, +P< 0.004).
Figure III: Recombinant CD40 ligand (rsCD40L) induces platelet Akt phosphorylation. Washed platelets were pre-incubated at 37°C with vehicle control, or PI3 kinase inhibitor, (LY29004, 25µM), p38 MAP kinase inhibitor (SB203580, 10µM), for 5 minutes in presence or absence of rsCD40L. Platelets were stimulated with TRAP (50µM) for an additional 3 minutes at 37°C, lysed, and analyzed by western blot as described in the methods section (a representative blot from 3 independent experiments).
**Figure IV**

<table>
<thead>
<tr>
<th></th>
<th>Phospho- p-38 MAPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet</td>
<td>+ + + + + + + + +</td>
</tr>
<tr>
<td>rsCD40L</td>
<td>- 2 10 - 10 - 10 10 (µg /ml)</td>
</tr>
<tr>
<td>TRAP</td>
<td>- - - + + + + +  +</td>
</tr>
<tr>
<td>SB 203580</td>
<td>- - - - + + - -</td>
</tr>
<tr>
<td>Ly29004</td>
<td>- - - - - - + +</td>
</tr>
</tbody>
</table>

Figure IV: Recombinant CD40 ligand (rsCD40L) induces platelet p-38 MAP kinase phosphorylation. Washed platelets were pre-incubated at 37° C with vehicle control, or p38 MAP kinase inhibitor (SB203580, 10µM), PI3 kinase inhibitor (LY29004, 25µM) for 5 minutes in presence or absence of rsCD40L. Platelets were stimulated with TRAP (50µM) for an additional 3 minutes at 37° C, lysed, and analyzed by western blot as described in the methods section (a representative blot from 3 independent experiments).
**Figure V**

Figure V: Inhibition of Akt phosphorylation significantly attenuates TRAP/rsCD40L induced platelet reactive oxygen and nitrogen species generation. Platelet stimulation induced RONS generation was determined using TRAP (20 μM) and rsCD40L (1 μg/ml) with flow cytometric monitoring of DCFHDA oxidation. Enhanced RONS generation are attenuated by PI3 kinase inhibitor Ly294002 (25 μM; n=3; \*P<0.005; **P<0.001).
Figure VI: Inhibition of p38 MAP kinase phosphorylation attenuates TRAP/rsCD40L induced platelet reactive oxygen and nitrogen species generation. Platelet stimulation induced RONS generation was determined using TRAP (20 µM) and rsCD40L (1 µg/ml) with flow cytometric monitoring of DCFHDA oxidation in presence and absence of p38 MAP kinase inhibitor SB203580 (10µM, n=3; *P<0.004; **P<0.001).
Figure VII

Platelets (a) + TRAP (b) TRAP + BAPTA (c)

Platelets + SB (d) +TRAP + SB (e) TRAP + SB + BAPTA (f)

Platelets (g) +TRAP (h) TRAP + rsCD40L (i)
+ rs CD40L + rsCD40L + BAPTA

Platelets + SB (j) TRAP + SB (k) TRAP + SB (l)
+ rs CD40L + rsCD40L + rsCD40L + BAPTA
Figure VII: Recombinant soluble CD40 Ligand (rsCD40L, 1µg/ml) enhances stimulation-induced platelet calcium mobilization. Fluorescence from fluo3-labeled platelets was inhibited by calcium chelator, Dimethyl bapta (10µM) but not by p38 MAP Kinase inhibitor SB203580 (10µM). Confocal images are presented at baseline (a, d, g, j) and after stimulation with 50µM TRAP in presence and absence of different additives (only 30 second time point is displayed; representative images from n=2).