Platelet CD40L Modulates Thrombus Growth Via Phosphatidylinositol 3-Kinase β, and Not Via CD40 and IκB Kinase α


Objective—To investigate the roles and signaling pathways of CD40L and CD40 in platelet–platelet interactions and thrombus formation under conditions relevant for atherothrombosis.

Approach and Results—Platelets from mice prone to atherosclerosis lacking CD40L (Cd40lg−/−Apoe−/−) showed diminished αmβ3 activation and α-granule secretion in response to glycoprotein VI stimulation, whereas these responses of CD40-deficient platelets (Cd40−/−Apoe−/−) were not decreased. Using blood from Cd40lg−/−Apoe−/− and Cd40−/−Apoe−/− mice, the glycoprotein VI-dependent formation of dense thrombi was impaired on atherosclerotic plaque material or on collagen, in comparison with Apoe−/− blood. In all genotypes, addition of CD40L to the blood enhanced the growth of dense thrombi on plaques and collagen. Similarly, CD40L enhanced glycoprotein VI–induced platelet aggregation, even with platelets deficient in CD40. This potentiation was antagonized in Pik3cbR/R−/− platelets or by inhibiting phosphatidylinositol 3-kinase β (PI3Kβ). Addition of CD40L also enhanced collagen-induced Akt phosphorylation, which was again antagonized by absence or inhibition of PI3Kβ. Finally, platelets from ChukIκB−/−Apoe−/− mice deficient in IκB kinase α (IκKα), implicated in CD40 signaling to nuclear factor (NF) κB, showed unchanged responses to CD40L in aggregation or thrombus formation.

Conclusions—Under atherogenic conditions, CD40L enhances collagen-induced platelet–platelet interactions by supporting integrin αmβ3 activation, secretion and thrombus growth via PI3Kβ, but not via CD40 and IκKα/NFκB. This role of CD40L exceeds the no more than modest role of CD40 in thrombus formation. (Arterioscler Thromb Vasc Biol. 2015;35:1374-1381. DOI: 10.1161/ATVBAHA.114.305127.)

Key Words: atherosclerosis • atherothrombosis • blood platelets • CD40 • CD40 ligand • signaling pathways • signal transduction • thrombosis

Atherothrombosis is a life-threatening clinical condition that can result in myocardial infarction or stroke because of thrombus formation on rupture or erosion of an atherosclerotic plaque.1 Both in vivo and in vitro studies have indicated that collagen is one of the main platelet-adhesive components of atherosclerotic plaques, causing platelet activation through the signaling collagen receptor, glycoprotein VI (GPVI).2-4 Collagen-dependent thrombus formation is a complex and highly orchestrated process. Key steps are the adhesion of platelets to collagen, the activation of flowing platelets into aggregates via secreted autocrine mediators, such as ADP and thromboxane A2; the activation of integrin αmβ3 via a phosphoinositide 3-kinase (PI3K) pathway to allow stable platelet–platelet interactions; and the formation of phosphatidylserine-exposing platelets, which support thrombin generation and fibrin formation.5

Platelets express substantial levels of the membrane glycoprotein CD40 (TNFR-SF5), which is an ubiquitous member of the tumor necrosis factor receptor (TNFR) superfamily.6,7 The alleged counter-receptor is CD40L (TNF-SF5 and CD154), which belongs to the TNF superfamily and is present on human platelets at ≈1600 copies/platelet.8 In activated platelets, CD40L can be cleaved from the membrane by extracellular proteases, yielding a functional, extracellular peptide that...
is usually indicated as soluble CD40L. Several reports point to a key role of platelet CD40L in atherosclerosis and atherothrombosis. Patients with (re)current cardiovascular disease often show increased levels of circulating soluble CD40L, predominantly originating from platelets. In mouse models, deficiency in Apoe or Ldlr leads to elevation of plasma lipids and predisposes for atherosclerosis. In these athereogenic mice, we and others have reported that additional deficiency in CD40L (Cd40lg−/−) or CD40 (Cd40−/−) markedly impairs plaque development. Furthermore, we have shown that the injection of activated Cd40lg−/− platelets to Apoe−/− mice suppresses platelet–leukocyte aggregate formation, subsequent inflammation, and the platelet-dependent progression of atherosclerosis.

The CD40L–CD40 axis may also play a role in the cross-talk of platelets themselves, and thus contribute to the stability of platelet thrombi. For instance, in mice deficiency in either CD40L or CD40 was found to protect against microvascular thrombosis when the animals were challenged by lipopoly saccharide. Studies have suggested a mechanism, in which CD40L interacts with CD40–TRAF2 and stimulates the classical nuclear factor (NF)κB pathway in platelets. In agreement with this postulation, pharmacological inhibition of NFκB was shown to suppress platelet activation. However, some reports indicate that the cleaved CD40L can interact with a different counter-receptor in both mouse and human platelets, namely the integrin β3 chain. The binding of CD40L can support αIIbβ3-dependent activation processes that have been implicated in thrombus stability.

In this article, we aimed to unravel the roles and the underlying signaling pathways of CD40L and CD40 in platelet activation and thrombus formation under conditions relevant for atherothrombosis. For this purpose, we used blood from Apoe−/− mice also deficient in CD40 or CD40L, prone to atherosclerosis, to investigate the activation pathways of platelets stimulated by collagen or plaque tissue via the GPVI receptor.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

To compare the roles of platelet CD40 and CD40L under conditions relevant for atherosclerosis and atherothrombosis, we used Apoe−/− mice with high plasma lipid levels and prone to atherosclerosis. Platelets were isolated from Apoe−/−, Cd40−/−Apoe−/−, and Cd40lg−/−Apoe−/− mice, and stimulated with key receptor agonists, that is, convulxin activating the collagen receptor GPVI, ADP as an agonist stimulating the P2Y1/P2Y12 receptors, and thrombin stimulating the PAR3/4 receptors. Platelet activation properties were compared by flow cytometry. Deficiency in CD40 led to increased integrin αIIbβ3 activation (measured with PE-JON/A monoclonal antibody) and increased α-granule secretion (measured with FITC (fluorescein isothiocyanate)-labeled anti-P-selectin monoclonal antibody), when the platelets were stimulated with a low or high dose of convulxin (Figure 1A and 1B). In marked contrast, deficiency in CD40L led to reduced αIIbβ3 activation and α-granule secretion after stimulation with convulxin. Responses triggered by thrombin or ADP were not changed in the CD40- or CD40L-deficient platelets. Thrombin, as a strong agonist, showed a rather steep dose–response curve with substantial integrin activation and P-selectin expression already at 0.25 nmol/L. At lower thrombin doses, these responses became highly variable (not shown). Stimulation with ADP resulted in high αIIbβ3 activation, but not in α-granule secretion, as previously demonstrated.

**Figure 1.** Agonist-induced integrin activation and secretion by Apoe−/− platelets lacking CD40 or CD40L. Washed platelets from Apoe−/−, Cd40−/−Apoe−/−, or Cd40lg−/−Apoe−/− mice were activated with the glycoprotein VI agonist convulxin (25–100 ng/mL), thrombin (0.25–2 nmol/L) or ADP (0.5–5 μmol/L). Using flow cytometry, activation of integrin αIIbβ3 was determined with PE-labeled JON/A monoclonal antibody (mAb; A), and P-selectin expression with FITC (fluorescein isothiocyanate)-labeled anti-CD62P mAb (B). Means±SEM (n=3–5), *P<0.05.
Cd40<sup>−/−</sup>Apo<sub>e</sub><sup>−/−</sup> and Cd40lg<sup>−/−</sup>Apo<sub>e</sub><sup>−/−</sup> platelets in comparison with Apoe<sup>+</sup> platelets (Figure 2D and 2E).

Blood perfusion over collagen gave comparable results: whereas platelet deposition was similar for each genotype, the build-up of large thrombi was consistently diminished in the absence of CD40L. This difference seemed most clearly from the area covered by multilayered aggregates, which was significantly lowered in the absence of CD40L, but not in the absence of CD40 (Figure 1A–IC in the online-only Data Supplement). Again, phosphatidylserine exposure was reduced in the absence of CD40 or CD40L, whereas P-selectin expression was unaltered (Figure ID and IE in the online-only Data Supplement).

To determine the ability of cleaved CD40L to support thrombus formation, blood samples from Apoe<sup>−/−</sup>, Cd40<sup>−/−</sup>Apo<sub>e</sub><sup>−/−</sup>, or Cd40lg<sup>−/−</sup>Apo<sub>e</sub><sup>−/−</sup> mice were preincubated with CD40L peptide before the perfusion. For all genotypes, this resulted in a substantial increase in thrombus formation on plaque material (Figure 2A) and on collagen (Figure IA in the online-only Data Supplement). Quantification learned that in the presence of CD40L peptide larger platelet aggregates were formed on plaque material (Figure 2C), which were best detected as

**Figure 2.** Increased thrombus formation on atherosclerotic plaque material by added CD40L in all genotypes. Blood from Apoe<sup>−/−</sup>, Cd40<sup>−/−</sup>Apo<sub>e</sub><sup>−/−</sup>, or Cd40lg<sup>−/−</sup>Apo<sub>e</sub><sup>−/−</sup> mice was anticoagulated with PPACK (H-d-phenylalanyl-prolyl-arginyl chloromethyl ketone)/heparin, and perfused over murine plaque material at 1000 s<sup>−1</sup> for 4 minutes. Blood samples were preincubated with CD40L (1 μg/mL), where indicated. Thrombi were stained by postperfusion with FITC (fluorescein isothiocyanate)-labeled anti-P-selectin monoclonal antibody and AF647-annexin A5.

A, Representative brightfield and fluorescence images (bar, 50 μm).

B, Quantification of surface area coverage (%SAC) of platelet deposition.

C, Quantification of integrated feature size from brightfield images. *P<0.05 compared with no CD40L, #P<0.05 compared with Apoe<sup>−/−</sup>.

D and E, Quantification of P-selectin expression and phosphatidylserine exposure from fluorescence images. Mean±SEM (n=3–5), *P<0.05.
multilayered aggregates on collagen (Figure 1F in the online-only Data Supplement), regardless of the genotype, that is, also with platelets deficient in CD40 or CD40L. Expression of P-selectin and phosphatidylserine exposure were not affected by the CD40L preincubation (not shown). Further experiments using blood from Apoe−/− mice indicated that preincubation of the blood with a blocking antibody against CD40L caused a reduction in the formation of multilayered aggregates showing less αIIbβ3 activation, thus indicating a role of autologous CD40L in the thrombus growth (Figure II in the online-only Data Supplement).

Other authors have reported a stimulating effect of the cleaved CD40L on platelet aggregation in response to low doses of agonists.18,19 To investigate this in more detail, we examined this in Apoe−/− mouse platelets also deficient in CD40L or CD40. Using platelets from Apoe−/−, Cd40−/−Apoe−/−, or Cd40lg−/−Apoe−/− mice, a low collagen dose (0.5 μg/mL) did not result in appreciable aggregation nor shape change, whereas a high dose (5 μg/mL) induced maximal aggregation for all genotypes (Figure 3A and 3B). Although exogenous CD40L alone was without effect, together with low collagen it caused near-maximal aggregation in all genotypes, including in platelets from the CD40-deficient mice. Taken together, these data point to a role of (cleaved) CD40L acting at least in part independently of CD40. In pilot experiments, we considered the possible involvement of another TNFR–TNF axis, that is, of the LIGHT receptor (TNFR-SF14) and LIGHT (TNF-SF14), which were reported to be present in platelets.26 However, a blocking antibody against this receptor was unable to inhibit the enhancement by CD40L of platelet responses (not shown).

Considering the major role of the PI3K/Akt signaling pathway in integrin activation,27 we compared the ability of collagen to induce phosphorylation at Ser473 of Akt in platelets from the CD40- and CD40L-deficient mice. The low dose of collagen (0.5 μg/mL) not causing platelet aggregation was also unable to stimulate Akt phosphorylation, whereas the high collagen dose (5 μg/mL) led to an increased phosphorylation by 4 to 6 times (Figure 3C). Markedly, this collagen-induced phosphorylation of Akt was diminished in Cd40lg−/−Apoe−/− platelets and tended to be lower in Cd40−/−Apoe−/−, when compared with Apoe−/− platelets (Figure 3D). Addition of CD40L alone did not evoke Akt phosphorylation (not shown), but in combination with low collagen, it caused a similar increase in phosphorylation in platelets from all genotypes (Figure 3C). Given that this phosphorylation is a prerequisite for integrin activation,28,29 these results suggest a key signaling role of PI3K-β in the stimulating effect of CD40L on collagen-induced platelet aggregation.

To further study the involvement of PI3K in CD40L-dependent platelet responses, we performed aggregation experiments with platelets from Pik3cbR/R mice, expressing a catalytically inactive K805R mutant of PI3K-β, that is, an isof orm that has previously been shown to control

Figure 3. Enhancing role of CD40L in collagen-induced platelet aggregation and Akt phosphorylation. Washed platelets (2.5×10⁸/mL) from Apoe−/−, Cd40−/−Apoe−/−, or Cd40lg−/−Apoe−/− mice were incubated with CD40L (1 μg/mL) for 30 minutes, and subsequently stimulated with the glycoprotein VI agonist, collagen (coll, 0.5–5 μg/mL). A, Representative aggregation traces. B, Maximal platelet aggregation (maximal change in light transmission). C and D, Platelet samples were taken from aggregation cuvettes after 10 minutes. Shown are representative western blots, and quantification of Akt phosphorylation at Ser473 and of total Akt. Lane 1: unstimulated platelets (unstim.), lane 2: collagen 5 μg/mL, lane 3: collagen 0.5 μg/mL, lane 4: collagen 0.5 μg/mL+CD40L. Bars indicate relative phosphorylation of Akt (relative to total Akt), compared with high collagen (C), or compared with unstimulated platelets (D). Mean±SEM (n=3–4), *P<0.05, #P=0.06.
collagen-dependent signaling events.\textsuperscript{30} When stimulated with high collagen, the aggregation of Pik3cb\textsuperscript{R/R} platelets was considerably reduced but not annulled (Figure 4A), which is in agreement with published results.\textsuperscript{31} With low collagen, exogenous CD40L failed to enhance the aggregation of Pik3cb\textsuperscript{R/R} platelets, in contrast to the large effect of CD40L seen with wild-type (Pik3cb\textsuperscript{WT/WT}) platelets (Figure 4A and 4B). Markedly, CD40L was also unable to stimulate Akt phosphorylation in Pik3cb\textsuperscript{R/R} platelets (Figure 4C). Additional confirmation for a role of PI3K-\(\beta\) came from the observation that the PI3K-\(\beta\)-specific inhibitor, TGX-221, abolished the stimulating effect of CD40L on collagen-induced aggregation (Figure III in the online-only Data Supplement).

Perfusion experiments were subsequently performed with blood from Pik3cb\textsuperscript{R/R} and Pik3cb\textsuperscript{WT/WT} mice over a collagen surface. In case of Pik3cb\textsuperscript{R/R} blood, the thrombi consisted of much smaller platelet aggregates, when compared with the wild-type (Figure IV in the online-only Data Supplement). For Pik3cb\textsuperscript{R/R} thrombi, P-selectin expression was unchanged, whereas phosphatidylserine exposure was reduced. Notably, addition of CD40L to blood samples increased the size of platelet aggregates for wild-type, but not Pik3cb\textsuperscript{R/R} mice. Confirming experiments were performed in the presence of TGX-221. When TGX-221-treated Apoe\textsuperscript{−/−} blood, was flowed over collagen or plaque material, this resulted in thrombi with smaller platelet aggregates and reduced phosphatidylserine exposure (Figure V in the online-only Data Supplement).

Largely based on pharmacological evidence, a role has been proposed for NF\(\kappa\)B pathways in CD40L- and CD40-mediated platelet responses.\textsuperscript{19} To take this further, we first studied effects of the NF\(\kappa\)B pathways in CD40L- and CD40-activated platelets. Markedly, in platelets from IKK\(\alpha\)-deficient mice, the ability of CD40L to enhance aggregation was not affected (Figure 5A). Flow studies further indicated that the size of platelet aggregates on collagen was not different for control Apoe\textsuperscript{−/−} and Chuk\textsuperscript{1\textalpha\textbeta}Apoe\textsuperscript{−/−} blood (Figure 5B–5D). Surprisingly, phosphatidylserine exposure was increased in thrombi from Chuk\textsuperscript{1\textalpha\textbeta}Apoe\textsuperscript{−/−} mice (Figure 5E). Together, these results indicate that the stimulatory effect of CD40L on thrombus growth is not mediated via the IKK\(\alpha\) pathway to NF\(\kappa\)B.

Discussion

The glycoproteins CD40L and CD40 play major roles in autoimmune and inflammatory reactions mediated by T-cells, endothelial cells, and leukocytes, by stimulating signaling pathways leading to activation of NF\(\kappa\)B.\textsuperscript{32} For the CD40L–CD40 dyad, involvement has been reported of the noncanonical and canonical NF\(\kappa\)B pathways.\textsuperscript{19} Cross-talk of CD40L and CD40 has also been shown for platelet–leukocyte and platelet–endothelial cell interactions and, as such implicated in the progression of mouse atherosclerosis\textsuperscript{13,15,38} and neoointima formation.\textsuperscript{16}
Here, we investigated the importance of the CD40L–CD40 axis for establishment of platelet–platelet interactions using mice with an atherogenic Apoe<sup>−/−</sup> background, that is, conditions where these platelet glycoproteins are considered to contribute to plaque progression. Our data indicate that CD40L and CD40 have, in part, different roles in platelet aggregation and thrombus growth.

In studies of whole blood thrombus formation on plaque material or collagen, in which platelet adhesion and activation are known to be triggered by GPVI, we found that deficiency in CD40L or CD40 (not significant with collagen) impaired the formation of large, multilayered platelet aggregates. This reduced thrombus growth did not coincide with altered α-granule secretion (P-selectin expression), but was accompanied by reduced phosphatidyserine exposure, an event that also is mediated by the collagen receptor GPVI. In accordance with these findings, others have reported that deficiency of CD40L alone (no Apoe<sup>−/−</sup>) leads to reduced arterial thrombus formation in vivo. In earlier work, we have found that deficiency of CD40 alone causes a small, insignificant reduction in platelet deposition on collagen. Together, these results point to a moderate contribution of CD40L and CD40 in collagen-dependent thrombus growth.

Flow cytometric studies gave, in part, similar results. When triggered with the GPVI agonist convulxin, platelets from Apoe<sup>−/−</sup> mice lacking CD40L showed a reduced integrin activation and secretion. However, platelets lacking CD40 responded by a higher integrin activation and higher P-selectin expression. The apparent different response of Cd40<sup>−/−</sup>Apoel<sup>−/−</sup> platelets, when compared with the measurement of thrombus formation, can be explained by the fact that in flow cytometry all platelets become activated by the added GPVI agonist, whereas during thrombus formation only those platelets in direct contact with the collagen are activated via GPVI and the other platelets respond to paracrine agents. Hence, it seems that in thrombus-forming experiments the apparent gain-of-function of Cd40<sup>−/−</sup>Apoel<sup>−/−</sup> platelets is overruled by non-GPVI–mediated secondary processes.

The dissociation seen in flow cytometry between the phenotypes of CD40L or CD40 deficiency is one indication for, in part, dissimilar roles of platelets CD40L and CD40. Further confirmation for a role of CD40L independently of CD40 in platelet–platelet interactions comes from (1) the observed thrombus-reducing effect by blocking CD40L, and (2) the enhanced platelet aggregation and thrombus formation noticed by supplementation of CD40L even for Cd40<sup>−/−</sup>Apoel<sup>−/−</sup> platelets lacking CD40. Hence, we could not reproduce the finding from others that knockout of CD40 abolishes CD40L-dependent platelet aggregation. The reason for this difference remains unclear. Taken together, our data imply that, in the stimulating platelet aggregation and thrombus growth, CD40L acts as least, in part, independently of CD40.

Another novel finding is the role of PI3K-β (using deficient mice and inhibitor TGX-221) and its downstream target Akt (based on Ser<sup>473</sup> phosphorylation) in the enhancing effect of CD40L on collagen-induced platelet aggregation and thrombus formation. This role of PI3K-β is in good agreement with the literature, demonstrating that this kinase is a major regulator of Akt phosphorylation and downstream integrin activation in response to various platelet agonists. Other authors have suggested that CD40L in platelets signals via p38 mitogen-activated protein kinase, or via tyrosine phosphorylation of the integrin β<sub>3</sub> chain. These pathways may coexist and help to support the establishment of platelet–platelet interactions. Of interest, a comparable signaling pathway, involving PI3K and Akt, has been proposed for endothelial cells, but in this case elicited by CD40 and leading to NFκB activation.

The unchanged collagen-induced aggregation and thrombus formation with Apoe<sup>−/−</sup> platelets deficient in IKKα activity
(Chuk\(^{1\alpha}\)), as well as the unchanged ability of CD40L to stimulate aggregation of these platelets, points to a no more than minor role of the noncanonical IKKα/NFκB pathway—whether or not triggered via CD40—in platelet–platelet interactions. In this respect, we like to note that evidence for a role of the NFκB pathway in CD40-dependent platelet function is largely based on the use of pharmacological inhibitors.\(^1\) We suspect that at least some of these inhibitors have effects on platelet responses (eg, Ca\(^{2+}\) signaling), that are not mediated via NFκB. However, several reports using IKKβ knockout mice point to a regulatory role of the canonical NFκB pathway in platelet secretion and glycoprotein shedding.\(^44,45\) Hence, we cannot rule out a role for this IKKβ/NFκB pathway in platelet activation responses evoked by CD40L independently of CD40.

Interestingly, in studying thrombus formation, we observed an increase in phosphatidylserine exposure of Chuk\(^{1\alpha}\)Apoe\(^{-/-}\) platelets, which we consider as further evidence that the IKKα/NFκB pathway does not contribute to the role of CD40L. Aκα platelets, which we consider as further evidence that the IKKβ/NFκB pathway is involved in platelet activation processes.\(^44,45\) Hence, we cannot rule out a role for this IKKβ/NFκB pathway in platelet activation responses evoked by CD40L independently of CD40.

Reports to date indicate that the cleavage of CD40L from activated platelets proceeds relatively slow and involves the matrix metalloproteinases 2 and 9, as well as integrin α\(^{\gamma}\)β\(^{3,9,10,46}\). Given these kinetics, our data suggest a role of both platelet-associated CD40L and cleaved CD40L in controlling thrombus growth. Taken together, we find that CD40L at least in part acting independently of CD40 is another of the (integrin-dependent) platelet-derived factors that contribute to the stable formation of large thrombi, especially under athrogenic conditions.

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### Disclosures

None.

### References


Significance

Under atherogenic conditions, CD40L enhances collagen-induced platelet–platelet interactions by supporting integrin αIIbβ3 activation, secretion, and thrombus growth via phosphoinositide 3-kinase β, but not via CD40 and IκB kinase α/nuclear factor κB. This role of CD40L exceeds the no more than modest role of CD40 in thrombus formation.
Platelet CD40L Modulates Thrombus Growth Via Phosphatidylinositol 3-Kinase β, and Not Via CD40 and IκB Kinase α

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Platelet CD40L modulates thrombus growth via phosphatidylinositol 3-kinase β, and not via CD40 or IKKα


Supplementary Figures

Supplementary Figure I. Reduced thrombus size on collagen in the absence of CD40 or CD40L is rescued by CD40L. Mouse blood anticoagulated with PPACK/heparin was perfused over collagen at 1000 s⁻¹ for 4 minutes. Pre-incubation with CD40L (1 µg/mL) was indicated. Thrombi were stained and images were analyzed as for Figure 2. A, Representative brightfield and fluorescence images (bar, 50 µm). B, Quantification of percentage surface area coverage (%SAC) with platelets (brightfield). C, Quantification of multi-layered thrombi from brightfield images. D, Quantification of effect of CD40L on integrated feature size. E-F, Quantification of surface area coverage of P-selectin expression and phosphatidylserine exposure from fluorescence images. Means ± SEM (n=3-6), *P<0.05, ***P<0.001, #P=0.06 vs. Apoe⁻/⁻ or absence of CD40L.

Supplementary Figure II. Inhibition of CD40L modulates thrombus growth on collagen. Blood from Apoe⁻/⁻ mice was anticoagulated with PPACK/heparin, supplemented with fibrinogen-AF546 (10 µg/mL) and perfused over collagen at 1000 s⁻¹ for 4 minutes. Blood samples were pre-incubated with a blocking antibody against murine CD40L (20 µg/mL) or control. A, Quantification of surface area covered by multi-layered thrombi (%SAC as percentage of control). B, Quantification of fluorescence images of AF546-fibrinogen. Means ± SEM (n=3), *P<0.05.
Supplementary Figure III. Inhibition of PI3K-β abrogates CD40L enhancement of collagen-induced platelet aggregation. Washed platelets from wildtype mice (2.5×10^8/mL) were pre-incubated with CD40L (1 µg/mL) and TGX-221 (1 µM), as indicated. Aggregation was induced with collagen (coll 0.5-5 µg/mL), and maximal amplitude of light transmission was measured. Means ± SEM (n=4-10), *P<0.05 compared to low collagen, **P<0.05 compared to low collagen + CD40L.

Supplementary Figure IV. Enforcing role of CD40L in thrombus formation on collagen via PI3K-β. Blood from Pik3cb^{R/R} or corresponding wildtype mice (Pik3cb^{WT/WT}) was perfused over collagen at 1000 s^{-1} for 4 minutes. Pre-incubation with CD40L (1 µg/mL) was as indicated. A, Representative brightfield and fluorescence images (bar, 50 µm). B, Quantification of integrated feature size. C-D, Quantification of P-selectin expression and phosphatidylserine exposure. Means ± SEM (n=3-4), *P<0.05 vs. Pik3cb^{WT/WT}.

Supplementary Figure V. Role of PI3K-β in thrombus formation on collagen or plaque material. Blood from Apoe^{-/-} mice was perfused over collagen or plaque material at 1000 s^{-1} for 4 minutes. Blood samples were pre-incubated with TGX-221 (1 µM) or vehicle (DMSO). Thrombi were stained and images were analyzed as for Figure 2. A, D, Representative brightfield and fluorescence images (bar, 50 µm). B, E, Quantification of integrated feature size from brightfield images. C, F, Quantification of phosphatidylserine exposure (%SAC). Means ± SEM (n=3-5), *P<0.05 vs. control.
**Supplementary Figure I**

A. Brightfield images of platelet deposition with 
- **α-P-Selectin-FITC**
- **Annexin A5-AF647**
- + CD40L

B. Bar graph showing platelet deposition (%SAC) for different genotypes:
- Apoe+/
- Cd40+/
- Cd40g+/

C. Bar graph showing multi-layered thrombi (%SAC) for different genotypes:
- Apoe+/
- Cd40+/
- Cd40g+/

D. Bar graph showing α-P-Selectin-FITC (%SAC) for different genotypes:
- Apoe+/
- Cd40+/
- Cd40g+/

E. Bar graph showing Annexin A5-AF647 (%SAC) for different genotypes:
- Apoe+/
- Cd40+/
- Cd40g+/

F. Bar graph showing integrated feature size (μm²) for different genotypes:
- Apoe+/
- Apoe+ + CD40L
- Cd40+/
- Cd40+ + CD40L
- Cd40g+/
- Cd40g+ + CD40L

Supplementary Figure I
Supplementary Figure II
Supplementary Figure III
Supplementary Figure IV
Supplementary Figure V

A. Collagen
Brightfield

Control

TGX-221

B. Brightfield FITC-Annexin A5

Control

TGX-221

C. Plaque
Brightfield

FITC-Annexin A5

Control

TGX-221

D. Brightfield FITC-Annexin A5

Collagen Plaque

E. Brightfield FITC-Annexin A5

Control

TGX-221

F. Brightfield FITC-Annexin A5

Control

TGX-221

* Significant difference
Platelet CD40L modulates thrombus growth via phosphatidylinositol 3-kinase β, and not via CD40 or IKKα


Materials and Methods

Materials
H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla CA, USA). Low molecular weight heparin (Fragmin) was from Pfizer (Capelle a/d IJssel, The Netherlands). Convulxin was purified to homogeneity from the crude venom of *Crotalus durissus terrificus* (Latoxan, Valence, France). Horm type I collagen was from Nycomed Pharma (Munich, Germany). Recombinant mouse CD40L peptide was from R&D Systems (Minneapolis MN, USA); enhancer for CD40L peptide from Enzo Life Sciences (Plymouth Meeting PA, USA); TGX-221, inhibitor of PI3Kβ, from Baker (Melbourne, Australia). NFκB pathway inhibitor Ro-106-9920, a selective inhibitor of the ubiquitination of activated IkBα, was from Sigma (St. Louis MO, USA). IKK inhibitor VII, an ATP-competitive inhibitor of IKK (targeting both IKK2/IKKβ and IKK1/IKKα) was from Calbiochem. Blocking antibody against murine CD40L (MR-1) was purified using protein G from hybridoma supernatant. Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD62P mAb, detecting P-selectin expression, and phycoerythrin (PE)-labeled anti-mouse JON/A mAb, detecting the activated conformation of integrin αIIbβ3, were from Emfret Analytics (Würzburg, Germany). Fibrinogen labeled with Alexa Fluor (AF) 546 was from Invitrogen Life Technologies (Carlsbad, CA, USA). Annexin A5 labeled with AF647 detecting phosphatidylserine exposure was from Molecular Probes (Eugene OR, USA). Antibodies against (phosphorylated) Akt were from Cell Signaling Technology (Danvers MA, USA). Ketamine and xylazine were obtained from Eurovet (Bladel, The Netherlands). Other materials were from sources as described before.
Animals
Experiments were approved by the local Animal Experimental Committees. Mice deficient in CD40 (Cd40<sup>-/-</sup>) or CD40L (Cd40lg<sup>-/-</sup>), all on Apoe<sup>−/−</sup> background, were obtained as described. Animals were included at age of 12-16 weeks, and were fed a chow diet. Generation of Pik3cb<sup>R/R</sup> mice with homozygous dysfunctional PI3K-β (Pik3cb gene), expressing a catalytically inactive K805R mutant of this enzyme, on Balb/c background is reported previously. Mice homozygous for activation-resistant mutation in IKKα (inhibitor of nuclear factor kappa-B kinase subunit α, also known as conserved helix-loop-helix ubiquitous kinase, Chuk1 gene) with a Ala replacements of Ser<sup>176/180</sup> in the kinase activation loop, were crossed with Apoe<sup>−/−</sup> mice to generate Chuk1<sup>A/A</sup> Apoe<sup>−/−</sup> mice, as described. Genotypes were confirmed by PCR from tail biopsies. CD40 genotyping was performed with the following primers: (a) CD40 12398: 5'-GTG AGATGCTAGCCCTCCTG-3', (b) CD40 12399: 5'-CACGTCATCTGGTTTTC-3' and (c) CD40 12400: 5'-CGTGCAATCCATCTTGTTCA-3'. Primers a and b yield a wildtype allele fragment of 594 bp and primers a and c give a mutant allele fragment of 685 bp. CD40L genotyping was performed with the following primers: (a) 1187 (mutant): 5'-GCCCTGAATGAATGCAGGACG-3', (b) 1188 (mutant): 5'-GGGTAGCCAACGCTATGTC-3', (c) 1189 (wildtype): 5'-GTTCCTCCACAATCCATCTTGTTCA-3' and (d) 1190 (wildtype): 5'-CCCAAGTGATGAGCATGTGTGT-3'. This yields a wildtype allele fragment of 250 bp and a mutant allele fragment of 500 bp. CD40L samples were separated and analyzed post-PCR by Quiaxcel (Qiagen).

Mouse atherosclerotic plaques were collected from the aortic arches of six Apoe<sup>−/−</sup> mice, fed with a Western-type diet with 0.25% cholesterol for 18-20 weeks. After resection, atherosclerotic specimens were frozen into liquid nitrogen and stored at -80 °C. Thawed mouse atherosclerotic tissues were homogenized in phosphate-buffered saline. Plaque homogenates were pooled at 165 mg wet tissue weight/mL, as described.

Blood collection and platelet preparation
Mice were anesthetized by subcutaneous injection of ketamine and xylazine (0.1 mg/g and 0.02 mg/g body weight) and blood was obtained by retro-orbital puncture. For flow studies, blood was collected into a triple anticoagulant solution of 40 µM
PPACK, 5 units/mL heparin and 40 units/mL Fragmin. For platelet preparation, blood was collected into one-sixth volume of acid-citrate-dextrose (ACD) anticoagulant (85 mM sodium citrate, 78 mM citric acid, 11 mM D-glucose). Washed platelets were prepared by centrifugation at 310 g for 3 minutes. Platelet-rich plasma (PRP) plus 1/3 red cells was transferred to a clean tube and centrifuged at 700 g for 1 second. PRP was removed, supplemented with ACD (1:15) and apyrase (0.1 unit/mL) and centrifuged at 1960 g for 5 minutes. The platelet pellet was then resuspended in modified Tyrode’s buffer (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH$_2$PO$_4$, 2 mM MgCl$_2$, 0.1% glucose and 0.1% bovine serum albumin, pH 7.45).

Cells were counted with a Coulter counter.

Mouse CD40L peptide (10 µg/mL) was pre-incubated with enhancer (2 µg/mL) for 30 minutes at room temperature. Washed platelets (2.5×10$^8$/mL) or whole blood was incubated with the mixture (1 µg/mL CD40L, f.c.) for 30 minutes at 37 °C, as described. In the absence of the enhancer, a two-fold higher concentration of CD40L peptide was needed for similar response enforcement.

**Flow cytometry**

Washed platelets (1×10$^8$/mL) in modified Tyrode’s buffer containing 2 mM CaCl$_2$ were activated with convulxin, thrombin or ADP without stirring. After 10 minutes, activation of integrin $\alpha_{IIb}\beta_3$ was detected with PE-labeled JON/A mAb (1:10), and $\alpha$-granule secretion with FITC-labeled anti-CD62 mAb (1:10). Fluorescence was measured with an Accuri C6 flow cytometer (Ann Arbor MI, USA).

**Platelet aggregation**

Light transmission aggregometry was performed with platelet-rich plasma or washed platelets, as described. When baseline was stable for 1-2 minutes, activation was started with indicated agonists. Aggregation measurements were stopped after 10 minutes, after which samples were lysed for western blot analysis.

**Western blotting**

Akt activation was measured by western blot analysis of platelet lysates. A polyclonal Ab against phosphoserine-473 Akt was used to detect the active form of Akt, and a polyclonal anti-Akt Ab to determine the total amount of Akt protein.
Thrombus formation under flow

Glass coverslips, coated with murine plaque material (170 µg wet weight/cm²) or fibrillar type I collagen (50 µg/mL), were blocked with modified Tyrode’s buffer containing 1% bovine serum albumin, and mounted into a transparent parallel-plate flow chamber, as described elsewhere.¹² PPACK/heparin/fragmin-anticoagulated blood was perfused through the chamber at a wall-shear rate of 1000 s⁻¹. After 4 minutes, the flow chamber was perfused with modified Tyrode’s buffer supplemented with 2 mM CaCl₂ and 1 unit/mL heparin at the same shear rate for 2 minutes. Phase-contrast images were taken from at least 10 microscopic fields, which were randomly chosen. Surface expression of activation markers on adhered platelets was measured after 5 minutes staining under stasis (EVOS microscope, Life Technologies, Carlsbad, CA, USA), i.e. expression of P-selectin using FITC-labeled anti-CD62P mAb and phosphatidylserine exposure with AF647-annexin A5.¹³ Images were analyzed with MetaMorph software (Molecular Devices, Sunnyvale CA, USA). Integrated feature size was calculated as a measure for the distribution of thrombi on the surface. For each image, the proportional contribution of large and small thrombi (i.e. features) to the total feature size was calculated.¹⁴ ¹⁵ Surface area coverage of multilayered thrombi was analyzed by outlining thrombi with more than one layer of platelets in each image.

Statistics

Significance of differences was determined with a parametric t-test or a non-parametric Mann-Whitney U test, as appropriate. The statistical package for social sciences was used (SPSS version 17, Chicago IL, USA). P values below 0.05 were considered statistically significant.

References


