CD40L stabilizes arterial thrombi by a β_3 integrin–dependent mechanism

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CD40L, a member of the tumor necrosis factor family of ligands, plays a major role in immune responses via its receptor, CD40. Recently, CD40L has been detected on the surfaces of activated platelets and shown to activate endothelium. Here we further addressed the function of platelet CD40L. We show that absence of CD40L affects the stability of arterial thrombi and delays arterial occlusion *in vivo*. Infusion of recombinant soluble (rs)CD40L restored normal thrombosis, whereas rsCD40L lacking the KGD integrin-recognition sequence did not. CD40-deficient mice exhibited normal thrombogenesis. rsCD40L specifically bound to purified integrin $\alpha_{IIb}\beta_3$ and to activated platelets in a β_3-dependent manner and induced platelet spreading. In addition, rsCD40L promoted the aggregation of either human or mouse platelets under high shear rates. Thus, CD40L appears to be an $\alpha_{IIb}\beta_3$ ligand, a platelet agonist, and necessary for stability of arterial thrombi.

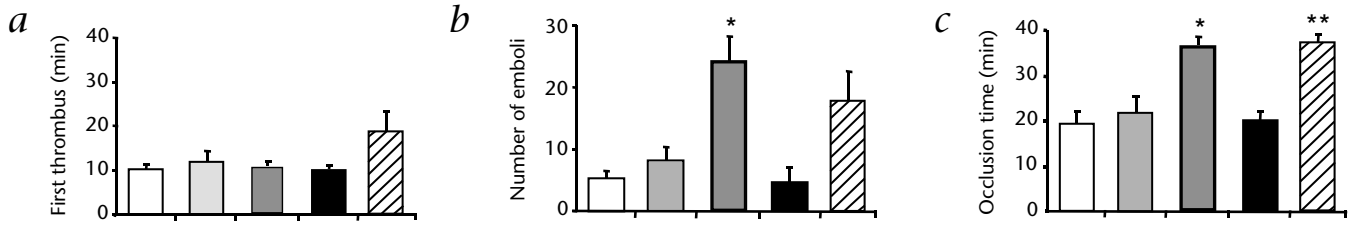
CD40L (also known as CD154 and gp39) is a transmembrane protein and member of the tumor necrosis factor (TNF) family. It is expressed on cells of the immune system (activated $CD4^+$ T cells, mast cells, basophils, eosinophils and natural killer cells)[1] and on activated platelets[2]. The receptor for CD40L, CD40, from the TNF receptor family, is widely distributed, primarily on cells of the vasculature. Several immune functions for CD40L have been reported; the most prominent is in isotype switching during the immune response. Mutations in the gene encoding CD40L lead to a human pathology termed X-linked hyper-immunoglobulin-M syndrome[3]. Ligation of CD40 by CD40L also has a role in the pathogenesis of atherosclerosis[4]. Although the etiology of these responses may result from immune deficiency, the inflammatory activity of CD40L on platelets and other cells within the vasculature has also been considered as CD40L induces the expression of chemokines (monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and IL-8), pro-inflammatory adhesion molecules (vascular cellular adhesion molecule-1, intracellular adhesion molecule-1 and P-selectin), and tissue factor (TF), and downregulates the expression of thrombomodulin in vascular cells[5–7].

CD40L is shed from stimulated lymphocytes generating soluble CD40L (sCD40L), and is actively released following platelet stimulation[8] and during acute coronary thrombosis[9]. A recent study shows that the proteolytic cleavage of CD40L from platelets is stimulated by its binding to CD40 which is expressed constitutively on platelets[8]. Although a precise role for sCD40L remains to be determined, two activities have been observed. First, sCD40L in serum of individuals with acute coronary thrombotic syndromes has been shown to be pro-inflammatory. In addition, sCD40L may promote coagulation as it induces TF expression on monocytes[10,11], similar to an activity of soluble P-selectin[12]. Soluble P-selectin in plasma promotes coagulation by causing the formation of TF-containing microparticles[13], and CD40L could have similar activity. sCD40L also contains a KGD sequence[14], a known binding motif specific for $\alpha_{IIb}\beta_3$ (ref. 15), the major platelet integrin. In addition, antibodies against CD40L induce thrombotic events in primates and humans[16]. Here we report a direct role for CD40L in high-shear platelet thrombosis that depends on a novel interaction of sCD40L with β_3 integrins in platelets.

Instability of large $CD40L^{-/-}$ thrombi in mesenteric arterioles

Thrombus formation in ferric chloride ($FeCl_3$)-induced injury of mesenteric arterioles of $CD40L^{-/-}$ mice was compared with that of wild-type mice using intravital microscopy[17,18]. Arterioles of 60–100-μm diameter with a shear rate of approximately 1300 per second were used. There was no difference in either initial adhesion of single platelets (97 ± 26 per min in $CD40L^{-/-}$ versus 101 ± 24 per min in wild-type mice) or in the times required for first thrombus growth in wild-type and $CD40L^{-/-}$ mice (Fig. 1*a*). However, in $CD40L^{-/-}$ mice, large thrombi frequently ruptured and embolized, an event rarely seen in wild-type vessels (Fig. 1*b*), leading to a delay in vessel occlusion (Fig. 1*c*). Histological examination of the thrombi formed showed lower platelet density in the absence of CD40L (Fig. 1*d*), which probably contributed to the fragility of the thrombi.

Platelets express 600–1,000 copies of CD40L (data not shown) and platelet activation is known to cause release of soluble CD40L (refs. 8,9). Therefore we investigated whether the soluble form of CD40L (rsCD40L) could restore the normal thrombotic process in $CD40L^{-/-}$ vessels. Infusion of 1.6 mg/kg of rsCD40L just



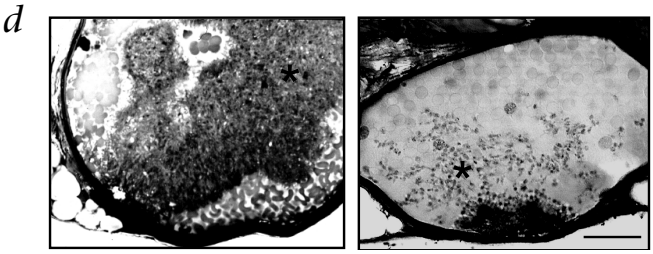


Fig. 1 Quantitative analysis of arterial thrombogenesis in wild-type, $CD40^{-/-}$ and $CD40L^{-/-}$ mice. ***a***, Time for appearance of first thrombus > 20 μm was similar in all groups. ***b***, The lack of CD40L significantly increased the number of thrombi > 30 μm that embolized before occlusion of the blood vessel (*$P < 0.0005$ compared with wild type). $CD40L^{-/-}$ thrombi grew to almost occlusion size but then ruptured and embolized in large pieces. Intravenous injection (i.v.) of rsCD40L, but not mrsCD40L(D117E), into $CD40L^{-/-}$ stabilized the thrombi. ***c***, The cyclic thrombotic process occurring in $CD40L^{-/-}$ mice significantly delayed versus wild type (*, $P < 0.0005$) the time for vessel occlusion; 2 of 8 $CD40L^{-/-}$ mice did not occlude by 40 min. The occlusion time was normalized by rsCD40L infusion but not by the mutant protein where 4 of 6 arteries did not occlude (**, $P < 0.01$ versus $CD40L^{-/-}$ + rsCD40L). No differences were observed between wild-type and $CD40^{-/-}$ mice. n = 5–8 mice. Wild type (□), $CD40^{-/-}$ (▩), $CD40L^{-/-}$ (■), $CD40L^{-/-}$ + rsCD40L (■), $CD40L^{-/-}$ + mrsCD40L(D117E) (▨). ***d***, Histological examination of the thrombi formed 30 min after $FeCl_3$-induced injury. Wild-type (left) arterioles revealed a densely packed platelet-rich thrombus, whereas a loosely packed luminal thrombus on top of a dense mural thrombus developed in the $CD40L^{-/-}$ (right) arteriole. Asterisk indicates luminal thrombus. Scale bar, 20 μm.

before injury both restored thrombus stability and reduced occlusion time to wild-type levels (Fig. 1*b* and *c*). No significant defects in thrombus formation were observed in $CD40^{-/-}$ mice (Fig. 1), indicating that CD40L is not acting through CD40 ligation. CD40L in which the KGD sequence was mutated to KGE, mrsCD40L(D117E), similarly prepared and tested for biological activity, did not restore normal thrombus formation in the $CD40L^{-/-}$ mice (Fig. 1). This finding shows that the single amino-acid mutation had a substantial effect on CD40L activity in platelet aggregation.

Normal hemostasis but impaired thrombosis in $CD40L^{-/-}$ mice

CD40L binding to CD40 is known to induce TF, a molecule promoting hemostasis[2,7,10]. We therefore evaluated the capacity of plasma from $CD40L^{-/-}$ and wild-type mice to form fibrin and quantified the amount of procoagulant plasma microparticles[13]. We did not find significant differences between $CD40L^{-/-}$ and wild-type mice either in plasma clotting time (4.6 ± 0.5 and 4.5 ± 0.5 min, respectively; n = 5) or in the level of plasma microparticles (4958 ± 282 and 4186 ± 497, respectively; n = 8). Thus, the observed instability of $CD40L^{-/-}$ thrombi (Fig. 1*b*) is probably not due to lack of fibrin formation[18]. We did not observe any defects in platelet aggregation *in vitro* at low shear rate with ADP, collagen, or thrombin as agonists (data not shown) nor significant prolongation in tail bleeding time in the $CD40L^{-/-}$ mice (113 ± 13 s versus 92 ± 12 s for the wild-type mice; n = 14, P = 0.12). To test whether the observed defect might be dependent on shear rate, a collagen-coated perfusion chamber was perfused with blood from $CD40L^{-/-}$ mice in the presence of hirudin; smaller thrombi formed at a 3,000 per second shear rate in $CD40L^{-/-}$ blood than in wild-type blood (Fig. 2). The initial platelet adhesion was normal (Fig. 2), indicating that the defect was in platelet–platelet interaction.

Because rsCD40L infusion restored normal thrombosis in $CD40L^{-/-}$ mice, we investigated whether rsCD40L could mediate or potentiate platelet aggregation *in vitro*. Washed $CD40L^{-/-}$ platelets activated with 10 μM ADP did not aggregate in the presence of 40 μg/ml rsCD40L in an aggregometer (shear rate ~100/s) (data not shown). On the other hand, washed $CD40L^{-/-}$ platelets stimulated with 0.2 U/ml thrombin and submitted 3 or 4 times to a high shear rate of 1,000 per second showed larger platelet aggregates in the presence of 40 μg/ml rsCD40L compared with incubation with vehicle buffer (Table 1). Next we tested whether the effects of CD40L on thrombus stability were mouse-specific or limited to $CD40L^{-/-}$ platelets. We individually examined blood treated with an anticoagulant from three human donors in a collagen-coated capillary perfusion chamber. We perfused the blood at shear rates of 200, 400 and 1,000 per second either alone or in the presence of rsCD40L or mrsCD40L(D117E). After embedding, sections were prepared and the thrombi areas and their length of adhesion with the substratum determined. As in the mouse thrombi, the platelet adhesion to the substratum was not enhanced by CD40L but the size of the thrombi, represented by their area on cross-section, was significantly increased (Fig. 3) at the high (1,000/s), but not at the lower shear rates (data not shown). mrsCD40L(D117E), did not affect thrombus growth at any shear rate. Thus, rsCD40L stabilized platelet aggre-

Table 1 Platelet aggregate size at high shear rate

	Thrombus area (mm^2)	
Number of passages	3	4
$CD40L^{-/-}$	829 ± 42	1790 ± 163
	932 ± 52	2090 ± 199
$CD40L^{-/-}$ + rsCD40L	3389 ± 1331	6565 ± 1166
	3112 ± 480	8473 ± 1714

Size of platelet aggregates formed at a 1,000/s shear rate. Washed $CD40L^{-/-}$ platelets stimulated with 0.2 U/ml thrombin and submitted 3 or 4 times to a high shear rate of 1,000/s in the stenosis chamber showed larger platelet aggregates (3–4 fold) in the presence of 40 μg/ml rsCD40L compared with incubation with buffer. Each value corresponds to the mean area covered by a platelet aggregate found in 20 μl of the platelet resuspension buffer. Data from 2 experiments/genotype/condition are presented.

a

	Surface covered with platelets (%)		Mean thrombus height(μm)	
Shear rate (/s)	645	3000	645	3000
Wild Type	39.8 ± 4.5	57 ± 1.9	17 ± 3.5	19.4 ± 1.7*
CD40L$^{-/-}$	40.7 ± 3.1	56.7 ± 3	18.5 ± 3	10.7 ± 2.1*

b

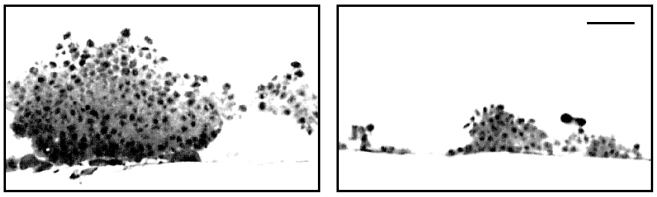


Fig. 2 Platelet deposition in a collagen-coated perfusion chamber. ***a***, Values were expressed as the mean ± s.e.m. of 3 separate experiments. Blood containing the thrombin inhibitor hirudin from 2 (645/s) or 3 (3000/s) mice was pooled for each experiment. Perfusion times were 3 min for 645/s and 2.25 min for 3000/s. Platelet adhesion (surface covered with platelets) was not affected by the absence of CD40L, but the thrombi formed at high shear rate were half the height of those in wild-type samples. *, $P < 0.05$. ***b***, Micrographs of thrombi formed at 3000/s. Left, wild type; right, CD40L$^{-/-}$. Scale bar, 10 μm.

gates *in vivo*, and enhanced platelet–platelet adhesion at high shear rate *in vitro* in a KGD-dependent manner.

Soluble CD40L binds platelets in a β_3 integrin-dependent manner

We investigated binding of rsCD40L to platelets by FACS analysis with an antibody specific for human CD40L that does not recognize the endogenous murine CD40L of wild-type platelets. rsCD40L did not bind to the surfaces of resting murine platelets. In contrast, platelets from wild-type or CD40$^{-/-}$ mice stimulated with 0.5 U/ml thrombin bound rsCD40L and this was significantly inhibited with the peptide GRGDSP, which is known to interfere with $\alpha_{IIb}\beta_3$-ligand binding (Table 2) but not with the control peptide GRGESP at the same 50 μM concentration (data not shown). We did not find binding of rsCD40L to thrombin-stimulated platelets from mice lacking β_3 integrin, which shows that binding of rsCD40L is dependent on the presence of this integrin (Table 2). CD40L binding to CD40$^{-/-}$ platelets was also reduced, indicating that CD40 may contribute to CD40L binding. However, absence of CD40 had no apparent effect on thrombus development and arterial occlusion *in vivo*.

Direct binding of rsCD40L to purified $\alpha_{IIb}\beta_3$

We evaluated binding of rsCD40L to purified $\alpha_{IIb}\beta_3$ and to purified CD40-Fc in a plate-binding assay. Specific binding of [^{125}I]-rsCD40L to $\alpha_{IIb}\beta_3$ occurred with a K_d of 30 ± 10 nM. (Fig. 4*a*). We also evaluated the relative activities of rsCD40L and mrsCD40L(D117E) in blocking [^{125}I]rsCD40L binding to $\alpha_{IIb}\beta_3$ and CD40. Although the two ligands had similar activities towards CD40 (85 ± 5% for mrsCD40L(D117E)), mrsCD40L(D117E) only inhibited [^{125}I]rsCD40L binding to $\alpha_{IIb}\beta_3$ by 49 ± 9% (P = 0.017 when compared with rsCD40L) (Fig. 4*b*).

$\alpha_{IIb}\beta_3$ integrin-dependent platelet spreading on rsCD40L

We also observed that rsCD40L deposited on glass induced spreading but not adhesion of resting human platelets (Fig. 5). Thus, rsCD40L, while not promoting platelet adhesion, activated the adherent platelets leading to their spreading. This phenomenon could be due to platelet stimulation by a direct rsCD40L–$\alpha_{IIb}\beta_3$ interaction as it was prevented by the inhibitors of $\alpha_{IIb}\beta_3$, Integrilin (Fig. 5) and abciximab (data not shown) and by mutating the KGD sequence in the rsCD40L (Fig. 5). The KGD sequence is specific for $\alpha_{IIb}\beta_3$, having low affinity for the minor platelet integrin $\alpha_v\beta_3$ (refs. 15,19).

Discussion

CD40L is crucial in T cell–dependent B-cell responses, and the CD40–CD40L interaction is a promising target of numerous therapeutic strategies. Interruption of the CD40–CD40L interaction has been shown to prolong allograft survival[20,21], and to limit the evolution of established atherosclerotic plaques in mice[22,23]. A recent report has also demonstrated that CD40–CD40L ligation promotes angiogenesis *in vivo*[24], and thus it could become a target of anti-angiogenic therapy. Here we provide evidence for a role for CD40L in thrombosis, extending the list of potential therapeutic targets involving CD40L.

In the *in vivo* injury model used in our study[18], CD40L$^{-/-}$ mice exhibited delayed vessel occlusion and frequent embolization compared with wild-type mice. Ligation of CD40 by CD40L is known to promote the expression of TF (ref. 5), and procoagulant leukocyte-derived microparticles expressing TF are a potent

a

	Mean thrombus area (μm^2)			Mean length of adhesion per thrombus (μm)		
	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
Untreated	17.7 ± 4	43.2 ± 3.7	40.9 ± 10	6.6 ± 0.9	6.9 ± 1.7	7.9 ± 2.6
+ rsCD40L	37.1 ± 4.6	69.6 ± 4.9	94.2 ± 11	6 ± 0.2	6.6 ± 0.7	6.5 ± 0.6
+ mrsCD40L(D117E)	18.6 ± 7.5	30.8 ± 9	37.1 ± 3.5	5.7 ± 0.6	4.4 ± 0.5	4.7 ± 0.4

b

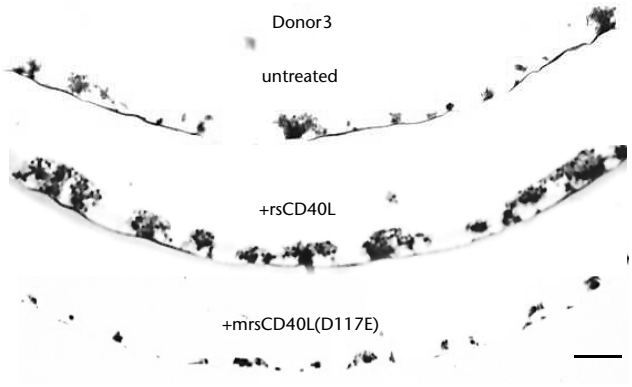


Fig. 3 Effect of rsCD40L on human thrombi growth at shear rate 1,000/s. Blood from 3 human donors was anticoagulated with PPACK and perfused through a capillary tube coated with type III collagen either alone (untreated) or containing rsCD40L or mrsCD40L(D117E). ***a***, Measurements of mean thrombus area and mean length of adhesion for all samples. ***b***, Photographs of a portion of the cross-section of the capillary tube showing thrombi obtained from donor 3. rsCD40L, but not the mutant protein, enhanced thrombus growth. Scale bar, 20 μm.

Table 2 Binding of rsCD40L to activated platelets

Genotype	Addition			
	–	rsCD40L	rsCD40L + GRGDSP	mrsCD40L (D117E)
Wild type	16.39 ± 2.3	41.1 ± 4.3[a,b,*]	12 ± 1.5	27.6 ± 5.6
$CD40^{-/-}$	13.8 ± 1.8	28.2 ± 3[b,**]	8.8 ± 1	22.6 ± 3.7
$\beta_3^{-/-}$	12.4 ± 3.6	12.9 ± 2.6[a]	ND	ND

Binding of rsCD40L to activated platelets (mean fluorescent intensity). Washed platelets were incubated with 0.5 U/ml thrombin and 40 μg/ml rsCD40L and with or without 50 μM RGD-containing peptide. Surface-bound soluble rsCD40L was revealed by FACS analysis using polyclonal anti-human CD40L and mean fluorescent intensity reported. Although the presence of β_3 was essential for rsCD40L binding to activated platelets ([a], $P < 0.005$), binding to $CD40^{-/-}$ platelets was also reduced ([b], $P < 0.03$) compared to wild type. Values represent mean ± s.e.m. of the mean fluorescent intensity of 3–9 experiments. *, In wild type mice, $P < 0.005$ versus untreated (-), or versus rsCD40L + GRGDSP; $P < 0.05$ versus mrsCD40L(D117E). **, In CD40–/– mice, $P < 0.02$ versus untreated (-); $P = 0.005$ versus rsCD40L + GRGDSP. ND, not determined.

source of fibrin deposition on activated platelets[13,25]. Furthermore, thrombosis in fibrinogen-deficient mice at high shear rate is characterized by unstable thrombi[18]. Therefore, we investigated whether these results could reflect a defect in thrombin/fibrin generation. Several lines of evidence showed that a platelet-function defect rather than a delay in thrombin/fibrin generation was responsible for thrombus instability in $CD40L^{-/-}$ mice. First, thrombi formed in the $CD40^{-/-}$ mice were stable (Fig. 1), showing that CD40 signaling is not involved. Second, both plasma clotting time and the number of plasma microparticles were similar in $CD40L^{-/-}$ and wild-type mice, indicating that CD40L does not affect the procoagulant potential of the mouse. Third, the *in vitro* perfusion chamber experiments did not involve thrombin or fibrin generation, and we still observed a defect in thrombosis of $CD40L^{-/-}$ blood (Fig. 2) and formation of larger thrombi with human blood treated with rsCD40L (Fig. 3).

Upon platelet activation, the transmembrane CD40L protein is exocytosed to the platelet plasma membrane from where it is also shed[2,8,9]. It is unclear whether both the transmembrane and the soluble forms of CD40L are active in promoting platelet activation and/or aggregation, but we have clearly demonstrated that rsCD40L alone can potentiate integrin-mediated platelet aggregation at high shear rates (Figs. 1 and 3 and Table 1). Notably, the platelet-shed CD40L seems to lose its endothelial activating potential[8]. It is therefore possible that cleavage of platelet CD40L reduces its proinflammatory activity while retaining or perhaps even increasing its prothrombotic effect.

Many integrin ligands, including those for $\alpha_{IIb}\beta_3$, use the RGD sequence for integrin recognition[26]. Although KGD is also an $\alpha_{IIb}\beta_3$ recognition motif[27], it has not been shown to be used by any of the known endogenous ligands. The presence of these motifs within the sCD40L structure (RGD in mouse; KGD in human) suggested a possible mechanism for its interaction with platelets. Using FACS analysis, we observed that rsCD40L bound wild-type and $CD40^{-/-}$ activated platelets and that this was significantly inhibited by GRGDSP (Table 2). Because no binding of rsCD40L occurred to thrombin-stimulated platelets from mice lacking β_3 integrins (Table 2), we performed experiments to determine whether rsCD40L bound directly to $\alpha_{IIb}\beta_3$. We observed specific saturable binding of $[^{125}I]$rsCD40L to human $\alpha_{IIb}\beta_3$. The K_d for CD40L and the $\alpha_{IIb}\beta_3$ integrin was approximately 30 nM, which is similar to that of fibrinogen (12 nM)[28]. As the local concentration of CD40L is unknown and may be high at the site of thrombus growth—where the protein becomes exposed on the plasma membrane or is shed—it is possible that CD40L can compete effectively with the other ligands for the binding to the integrin. We also observed that immobilized rsCD40L induced platelet spreading, which was blocked by inhibitors of $\alpha_{IIb}\beta_3$. These results indicated that rsCD40L bound directly to $\alpha_{IIb}\beta_3$, and that ligand binding induced platelet stimulation.

Ligands that use the RGD recognition motif characteristically show reduced affinity when any of its three amino acids are mutated. KGD is a specific $\alpha_{IIb}\beta_3$-recognition motif, and mutations in this motif (for example, a D-to-E point mutation) similarly cause a decrease in integrin affinity[15]. We have demonstrated that sCD40L with a mutation in its KGD sequence has reduced competitive binding activity towards $\alpha_{IIb}\beta_3$ while retaining activity towards CD40. As $CD40^{-/-}$ mice had normal thrombogenesis (Fig. 1), and the (D117E) mutation in rsCD40L abolished both the effect of CD40L on platelet spreading and thrombus growth *in vitro* (Figs. 3 and 5) and the thrombus-stabilizing activity of rsCD40L *in vivo* (Fig.1), we concluded that rsCD40L acts via the KGD sequence. The mechanism for rsCD40L-induced thrombus stability is presently unknown, but there are several possibilities. The KGD sequence may directly activate the ligand binding activity of $\alpha_{IIb}\beta_3$ as reported for RGD peptide[29]. The CD40L in its trimeric form may also cluster the integrins leading to platelet activation or directly cross-link the platelets in high shear environments. Further work is needed to clarify this issue. As CD40L binds the platelet β_3 integrin, it may be a ligand of β_3 and/or of additional integrins in other cellular interactions as well.

Our study extends the list of biological activities of CD40L from immune responses and atherosclerosis to platelet activation and thrombosis. Although lack of CD40L may be associated with thrombus fragility, high levels of soluble CD40L, which have been reported in patients with unstable angina[9], could constitute an increased risk factor for thrombosis especially in regions of stenosis (high shear rate). Antibodies to CD40L have been used in

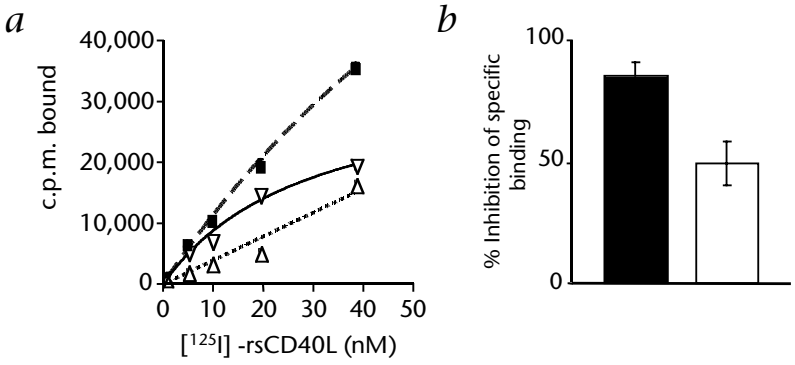


Fig. 4 rsCD40L binding to $\alpha_{IIb}\beta_3$. ***a***, Specific binding (▽) of $[^{125}I]$rsCD40L to immobilized purified $\alpha_{IIb}\beta_3$ was determined by subtracting the non-specific binding (△) from total binding (■). A representative of 3 experiments performed in triplicate is shown. c.p.m., count per minute. ***b***, 100-fold excess of mrsCD40L(D117E) fully inhibited specific binding of $[^{125}I]$rsCD40L to CD40 (■) but inhibited specific binding of $[^{125}I]$rsCD40L to $\alpha_{IIb}\beta_3$ only partially (□). Specific binding of $[^{125}I]$rsCD40L to both $\alpha_{IIb}\beta_3$ and CD40 was determined by the addition of 100-fold excess unlabeled rsCD40L.

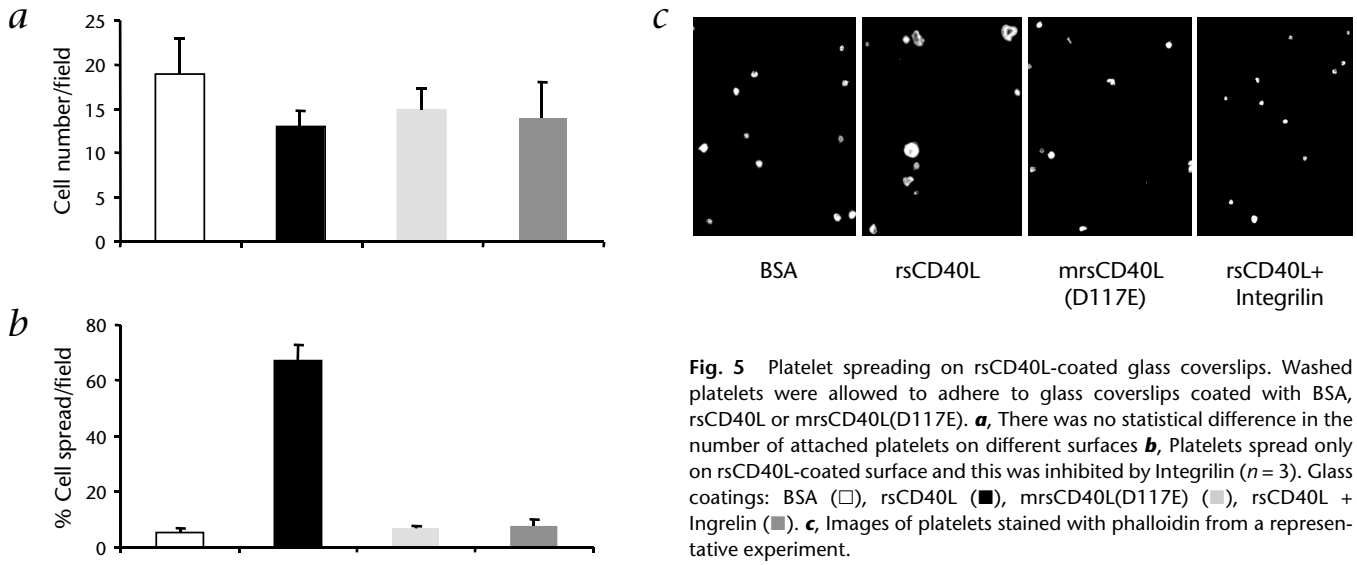


Fig. 5 Platelet spreading on rsCD40L-coated glass coverslips. Washed platelets were allowed to adhere to glass coverslips coated with BSA, rsCD40L or mrsCD40L(D117E). ***a***, There was no statistical difference in the number of attached platelets on different surfaces ***b***, Platelets spread only on rsCD40L-coated surface and this was inhibited by Integrilin ($n = 3$). Glass coatings: BSA (□), rsCD40L (■), mrsCD40L(D117E) (▩), rsCD40L + Ingrelin (▩). ***c***, Images of platelets stained with phalloidin from a representative experiment.

clinical trials and in animal models of human disease[20–23]. Interestingly, some thromboembolic complications were reported in both animals and humans[16]. These could result from the interaction of the antibody with the platelet Fc receptors, but our results suggest that inhibition of platelet CD40L might also render platelet plugs unstable (ready to embolize). However, inhibition of CD40L could help to prevent formation of stable thrombi when this is not desired; for example, after a cardiovascular intervention to prevent restenosis. Thus the clinical outcomes of anti CD40L therapy will have to be carefully evaluated.

Methods

Mice. Wild-type, CD40L$^{-/-}$ (Immunex, Seattle, Washington) and CD40$^{-/-}$ mice[30], all of C57BL/6J background, were obtained from the Jackson Laboratory (Bar Harbor, Maine). Integrin β_3-deficient mice were described[31]. Animals were housed at the Center for Blood Research, and experimental procedures were approved by its Animal Care and Use Committee.

Cloning and expression of human rsCD40L. A cDNA of human sCD40L (aa 108–261) was prepared by reverse transcribing polyA RNA isolated from Jurkat cells (ATCC, Bethesda, Maryland) using a forward primer (5′-CGAATTCCT CTTCCATGGAAAACAGCTTTGAAATG-3′) and a reverse primer (5′-GACTC TTCGAAGCTAGGATCCTAGGGTTA-3′) for CD40L. cDNA was cloned into pDUAL vector (Stratagene, La Jolla, California), protein expressed in BL23(DE3)-RIL (Stratagene). Protein was purified as described[32]. Fractions containing rsCD40L were pooled, concentrated and applied to Sephacryl S-100 (Amersham, Piscataway, New Jersey), then to Q-Sepharose (Amersham). Protein was passed through a Detoxi-Gel column (Pierce-Endogen, Rockford, Illinois). Mutant rsCD40L carrying a point mutation (mrsCD40L(D117E)) was created by oligo-based PCR and purified as above. Purified proteins were sequenced at the microchemical facility, Emory University School of Medicine, Atlanta, Georgia, confirming human rsCD40L (aa108–261) and mrsCD40L(D117E).

Biological function of bacterially expressed rsCD40L. HUVECs (Clonetics, San Diego, California), passage 3-5, grown in 6-well dishes coated with 0.75% bovine skin gelatin (Sigma, St. Louis, Missouri) were incubated with and without 2 μg/ml rsCD40L in the presence of TRAP-1 (anti-human CD40L antibody, BD PharMingen, San Diego, California) or MOPC-21 (mouse IgG, Sigma) for 4 h at 37 °C in Tyrode's buffer containing 1 mM $CaCl_2$ and 1mM $MgCl_2$. Concentration of MCP-1 was determined by ELISA (Pierce-Endogen). HUVECs exposed to rsCD40L showed a 3-fold increased secretion of MCP-1 (8.4 ± 1.4 ng/ml versus buffer treated cells 2.64 ± 0.03 ng/ml). This increase was blocked by TRAP-1 (2.6 ± 0.08 ng/ml).

Intravital microscopy. Platelets were isolated from platelet-rich plasma (PRP) and labeled as described[33]. Fluorescent platelets (5×10^9 platelets/kg) were infused into mice (3- to 4-wk-old) of matching genotypes. A mesenteric arteriole was chosen and injured with ferric chloride ($FeCl_3$, 30 μl of a 250 mM solution)[17,18]. Some CD40L$^{-/-}$ mice were injected i.v. with 1.6 mg/kg rsCD40L or mrsCD40L(D117E), 5–10 min before $FeCl_3$ injury. The tapes were analyzed by an investigator blinded to the genotype and treatment.

Hemostatic parameters. Tail bleeding time[17], plasma clotting time assay and flow cytometry determination of plasma microparticles were done as described[13]. For platelet aggregation induced by ADP (10 μM) or collagen Horm (5 μg/ml, Nycomed, Munich, Germany), platelet count in PRP was adjusted to 3×10^8 platelets/ml with autologous platelet-poor plasma (PPP). Washed platelets (3×10^8 platelets/ml) were used for aggregation induced by thrombin. Platelet aggregation was performed at 1,000 revolutions per minute at 37 °C, using a lumi-aggregometer (Sienco, Wheat Ridge, Colorado).

Perfusion chamber used with mouse blood. A silicone gasket with a flow-path height of 127 μm was placed between a flat perfusion chamber (Glycotech, Rockville, Maryland) and a human type III fibrillar collagen-coated Petri dish (Sigma). Shear rates of 3,000/s, and 645/s, were generated with flow rates of 1.2, and 0.26 ml/min, respectively, of hirudin-treated (160 U/ml, Sigma) blood. Measurements of thrombotic deposits were described[34].

Perfusion chamber for human blood. Blood was collected on PPACK (final concentration, 40 μmol/L). The blood sample was divided into 3 tubes (untreated, 40 μg/ml rsCD40L, and 40 μg/ml mrsCD40L(D117E)). Blood was perfused through glass capillary tubes (0.56 mm inner diameter) coated with 1 mg/ml type III collagen (Sigma)[34], at 1 ml/min for 3 min (1,000/s) and chamber processed for embedding and analysis[34,35].

Aggregation of thrombin-stimulated platelets under high shear rates. 400 μl of washed CD40L$^{-/-}$ platelets were activated with 0.2 U/ml human thrombin and incubated with 40 μg/ml rsCD40L or vehicle buffer for 30 min at 37 °C. Platelets were then perfused 3 or 4 times through a 1% albumin-coated perfusion chamber (127 μm height) at 400 μl/min (1,000/s), fixed, deposited on a coverslip and the mean thrombi area quantified using the Leica Q500MC image analysis program (Leica, Deerfield, Illinois).

Flow cytofluorometry study of rsCD40L-platelet binding. Washed platelets were activated with 0.5 U/ml human thrombin in presence of 40 μg/ml rsCD40L for 30 min at 37 °C. In some experiments, 50 μM GRGDSP or GRGESP (Invitrogen, Carlsbad, California) was added. Samples were then incubated for 30 min at room temperature with a rabbit polyclonal anti-

human CD40L that does not recognize endogenous CD40L of wild-type platelets (1:500 dilution, COR Therapeutics) or Rabbit IgG (1:500 dilution, Sigma), stained with a FITC-conjugated goat-anti rabbit IgG (1:500 dilution, Organon Teknika, Durham, North Carolina), and analyzed by flow cytometry.

rsCD40L binding assay. Nunc-Maxisorp 96-well plates were coated overnight at 4 °C with 5 μg/mL of purified $\alpha_{IIb}\beta_3$ or CD40-Fc (Alexis Biochemicals, San Diego, California) and blocked with 35 mg/ml BSA. [^{125}I]rsCD40L (labeled by IODO-bead method, Pierce-Endogen) was added in Buffer A (0.1M NaCl, 0.05 M Tris (pH 7.4), 0.2 M $CaCl_2$ and 10 mg/mlBSA) and incubated for 2 h at room temperature to determine total binding to $\alpha_{IIb}\beta_3$ or CD40. Non-specific binding was determined by measuring [^{125}I]rsCD40L in the presence of 100-fold molar excess of unlabeled ligand. The amount of radioactive ligand bound was determined after 3 washes with Buffer A. K_d was calculated by plotting the data using Graphpad software (Graphpad Prism, San Diego, California).

Platelet-spreading assay. Washed platelets were prepared[36] but 50 ng/ml of PGI_2 was present during blood collection and in the first wash. Coverslips were coated with 10 μg/ml crystalline BSA (Sigma) or rsCD40L for 2 h at 37 °C, and blocked with 5% crystalline BSA. 100 μl of 1×10^8 platelets/ml with or without 20 μM integrilin were allowed to spread for 1 h at 37 °C. Platelets in suspension were discarded, adherent cells were fixed, washed and permeabilized with 0.5% Triton X-100 and 0.5% BSA for 15 min. Cells were incubated for 45 min with 1:50 of Alexa Fluor 546 phalloidin (Molecular Probes, Eugene, Oregon), washed and mounted on slides with VECTASHIELD (Vector Laboratories, Burlingame, California). Total numbers of cells attached or spread were counted in 3 randomly selected fields (oil, ×65) per coverslip using a Zeiss Axiovert inverted fluorescence microscope (S100) and photographed using a spot 2 camera (Diagnostic Instruments, Sterling Heights, Michigan).

Statistics. Statistical analysis was performed using the unpaired Student's *t*-test.

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Competing interests statement.

The authors declare competing financial interests: see the website (http://medicine.nature.com) for details.

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