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Involvement of nuclear factor κB in platelet CD40 signaling

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ABSTRACT

CD40 ligand (CD40L) is a thrombo-inflammatory molecule that predicts cardiovascular events. Platelets constitute the major source of soluble CD40L (sCD40L), which has been shown to potentiate platelet activation and aggregation, in a CD40-dependent manner, via p38 mitogen activated protein kinase (MAPK) and Rac1 signaling. In many cells, the CD40L/CD40 dyad also induces activation of nuclear factor kappa B (NF-κB). Given that platelets contain NF-κB, we hypothesized that it may be involved in platelet CD40 signaling and function. In human platelets, sCD40L induces association of CD40 with its adaptor protein the tumor necrosis factor receptor associated factor 2 and triggers phosphorylation of IκBα, which are abolished by CD40L blockade. Inhibition of IκBα phosphorylation reverses sCD40L-induced IκBα phosphorylation without affecting p38 MAPK phosphorylation. On the other hand, inhibition of p38 MAPK phosphorylation has no effect on IκBα phosphorylation, indicating a divergence in the signaling pathway originating from CD40 upon its ligation. In functional studies, inhibition of IκBα phosphorylation reverses sCD40L-induced platelet activation and potentiation of platelet aggregation in response to a sub-threshold concentration of collagen. This study demonstrates that the sCD40L/CD40 axis triggers NF-κB activation in platelets. This signaling pathway plays a critical role in platelet activation and aggregation upon sCD40L stimulation and may represent an important target against thrombo-inflammatory disorders.

1. Introduction

CD40 is a 48 kDa membrane glycoprotein belonging to the tumor necrosis factor (TNF) receptor family. It was first discovered on human bladder carcinoma cells [1], but is now known to be present on a plethora of cell lines including B lymphocytes, endothelial cells, monocytes, dendritic cells and platelets [2–5]. Interaction of CD40 with its CD40 ligand (CD40L), a member of the TNF superfamily, plays a pivotal role in the immune response. The cytoplasmic domain of CD40 lacks direct kinase activity and therefore utilizes members of the TNF receptor-associated factors (TRAFs) as adapter proteins to mediate signaling events. TRAF1, 2, 3, 5 and 6 have been shown to interact with the cytoplasmic domain of CD40 and regulate downstream signaling pathways upon its ligation [6–8]. TRAF6 and TRAF2 mediate the activation of the canonical and non-canonical NF-κB pathways in response to CD40 engagement.

Nuclear factor κB (NF-κB) proteins are formed by hetero- or homo-dimerization of the five Rel/NF-κB DNA-binding subunits, which include RelA (p65), RelB (p68), c-Rel, p50 (NF-κB1) and p52 (NF-κB2). These NF-κB complexes are maintained in the cytoplasm in an inactive state through the inhibitor κB (IκBα or IκBβ). In response to stimuli, the NF-κB dimers in association with the inhibitory IκB subunit are regulated by the IκB kinase (IKK), which consists of two kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO. Upon phosphorylation by IKK, the IκB subunit is targeted for proteosomal degradation, thereby releasing an active form of NF-κB that translocates into the nucleus. Nuclear translocation of p50/RelA and p52/RelB is responsible for the canonical and non-canonical NF-κB pathways, respectively [9]. Liu et al. [10] has demonstrated the presence of NF-κB and IκBα in platelets, as well as the induction of the NF-κB signaling pathway following platelet activation. Thereafter, Malaver et al. [11] has shown that IκBα is phosphorylated in thrombin-activated platelets and pharmacological inhibition of this factor leads to impairment of platelet function, thereby attributing non-genomic functions to NF-κB in anucleated platelets. In contrast, Gambaryan et al. [12] showed that NF-κB negatively regulates platelet activation by thrombin and collagen via PKAc activation.

Abbreviations: TNF, tumor necrosis factor; sCD40L, soluble CD40 ligand; TRAF, tumor necrosis factor receptor-associated factor; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; JNK, c-jun amino terminal kinase; ERK, extracellular signal-regulated protein kinase; SNAP-23, synaptosomal-associated protein 23.

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Platelets are pivotal contributors to thrombosis and homeostasis, but also participate in inflammation and immunity [13]. The work by Henn et al. [5,14] demonstrated the presence of the CD40L/CD40 dyad in platelets, they showed that CD40 is constitutively expressed on the platelet surface, while CD40L rapidly appears on the platelet surface following activation. Surface expressed CD40L is subsequently cleaved into an 18 kDa fragment, which accounts for >95% of plasmatic soluble CD40L (sCD40L) concentrations [15]. Circulating levels of sCD40L in patients have now emerged as strong indicators of cardiovascular risk, as there appears to be a significant correlation between elevated levels of sCD40L and vascular complications such as atherosclerosis and acute coronary syndromes [16–18].

We have previously shown that sCD40L enhances agonist-induced platelet activation and aggregation through a CD40-dependent TRAF2/Rac1/p38 mitogen-activated protein kinase signaling (MAPK) pathway [19]. Although the presence of both the CD40L/CD40 dyad and NF-κB/IκBα in platelets is recognized, the involvement of NF-κB/IκBα in platelet CD40 signaling and function remains unknown. This study was therefore designed to test the hypothesis that NF-κB is involved in platelet CD40 signaling and function.

2. Materials and methods

2.1. Reagents and antibodies

Recombinant human sCD40L was obtained from R&D systems. Antibodies against TRAF2 (rabbit polyclonal), phospho-IκBα (mouse monoclonal, $Ser^{32/36}$), phospho-p38 MAPK (rabbit polyclonal, Thr^{180}/Tyr^{182}) and β-actin (rabbit polyclonal) were purchased from Cell Signaling Technology. The mouse monoclonal anti-CD40 antibody used for immunoprecipitation of human CD40 was also from R&D systems, while the rabbit polyclonal anti-CD40 antibody used for detection of CD40 by immunoblotting came from Santa Cruz Biotechnology. Antibody against CD62P (mouse monoclonal, AK4-PE conjugated) was obtained from BD Biosciences. The specific IKK inhibitor VII and the p38 MAPK inhibitor SB203580 were purchased from Calbiochem, while the IκBα phosphorylation inhibitor BAY 11-7082 was purchased from Sigma–Aldrich. Protein A agarose beads were obtained from Upstate Biotechnology, Inc. Native type I collagen was from Chronolog Corp.

2.2. Platelet isolation

Venous blood was drawn from healthy volunteers, free from medication known to interfere with platelet function for at least 10 days before the experiment. The protocol was approved by the human ethical committee of the Montreal Heart in accordance with the declaration of Helsinki for experiments involving humans. Washed platelets were prepared as previously described [19], adjusted to the indicated concentrations and allowed to rest at 37 °C for 30 min before further manipulation.

2.3. Flow cytometry

Platelet P-selectin (CD62P) expression, as a marker of α granule secretion and platelet activation, was measured by flow cytometry as previously described [19]. Briefly, platelets (250×10^6/mL) were pre-incubated with or without the indicated inhibitor for 10 min at 37 °C. Platelets were then stimulated with sCD40L for 30 min at 37 °C, fixed with 1% paraformaldehyde, washed and stained with saturating concentrations of anti-CD62P antibody for 30 min or its isotype-matched control IgG. Platelets were analyzed (20,000 events) on an Altra flow cytometer (Beckman Coulter) after gating their characteristic forward and side scatter properties.

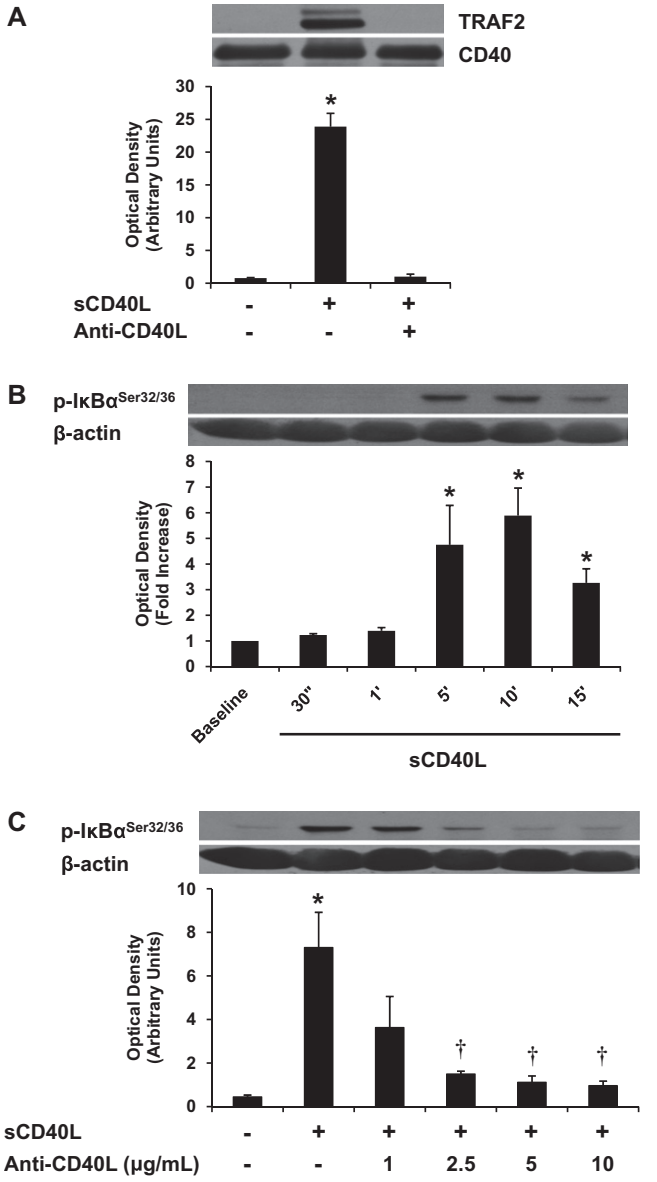

Fig. 1. sCD40L induce TRAF2 association to CD40 and IκBα phosphorylation in platelets. (A) Platelets (500×10^6/mL) were left untreated or incubated with 1 μg/mL sCD40L for 15 min at 37 °C in the presence or absence of a blocking anti-CD40L antibody (5 μg/mL). Total cell lysates were then immunoprecipated using an anti-CD40 antibody and immunoblotted for TRAF2 and CD40 expression. Blots are representative of three independent experiments. Histogram represents the mean of data of overlay blots, expressed as arbitrary units of optical density ($n = 3$; $^*P < 0.05$ vs. baseline or sCD40L). (B) Time-dependent course of IκBα phosphorylation on $Ser^{32/36}$ (p-IκBα $Ser^{32/36}$) following sCD40L treatment. Lysates form 1000×10^6 platelets/mL from untreated (Baseline) or sCD40L-treated (1 μg/mL) were resolved in 12% SDS–PAGE and assessed for p-IκBα $Ser^{32/36}$. Blots are representative of three independent experiments. Histogram represents the mean of data of overlay blots, expressed as fold increase in optical density, as compared to baseline ($n = 3$, $^*P < 0.05$ vs. baseline). (C) Dose-dependent effect of anti-CD40L treatment on IκBα phosphorylation. Platelets were left untreated or pretreated with the indicated dose of anti-CD40L for 5 min at 37 °C prior to stimulation with sCD40L. Platelet lysates were then analyzed for p-IκBα $Ser^{32/36}$. β-Actin blots shown are from stripped p-IκBα $Ser^{32/36}$ membranes. Blots are representative of five independent experiments. Histogram represent the mean of data of overlay blots, expressed as arbitrary units of optical density ($n = 5$, $^*P < 0.05$ vs. baseline and $^\dagger P < 0.05$ vs. sCD40L alone).

2.4. Platelet aggregation

Platelets were adjusted to 250×10^6/mL and aggregation was monitored on a four-channel optical aggregometer (Chronolog Corp.) under shear (1000 rpm) at 37 °C. Platelets were pre-incubated with or without the indicated inhibitors for 10 min at 37 °C prior to incubation with sCD40L for an additional 30 min under static conditions at 37 °C. Platelet aggregation was then monitored following the addition of collagen (0.5 μg/mL) and recorded until stabilization of platelet aggregation.

2.5. Immunoprecipitation of CD40

Platelets were stimulated as indicated and lysed into ice-cold modified RIPA lysis buffer (1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mM sodium-orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 2 μg/mL benzamidin) for 1 h at 4 °C. Lysates were sonicated on ice and pre-cleared with 100 μL of protein A agarose beads for 15 min at 4 °C. Beads were then pelleted and the supernatant was incubated with an anti-CD40 antibody overnight at 4 °C. Samples were treated with 100 μL of protein A agarose beads for 1 h at 4 °C. Beads were then precipitated by centrifugation, washed three times with ice-cold modified RIPA lysis buffer, resuspended in 2X Laemmli buffer and boiled for 5 min. Supernatants were analyzed by immunoblotting for the presence of TRAF2 and CD40 proteins.

2.6. SDS–PAGE and immunoblotting

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

2.7. Statistical analysis

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

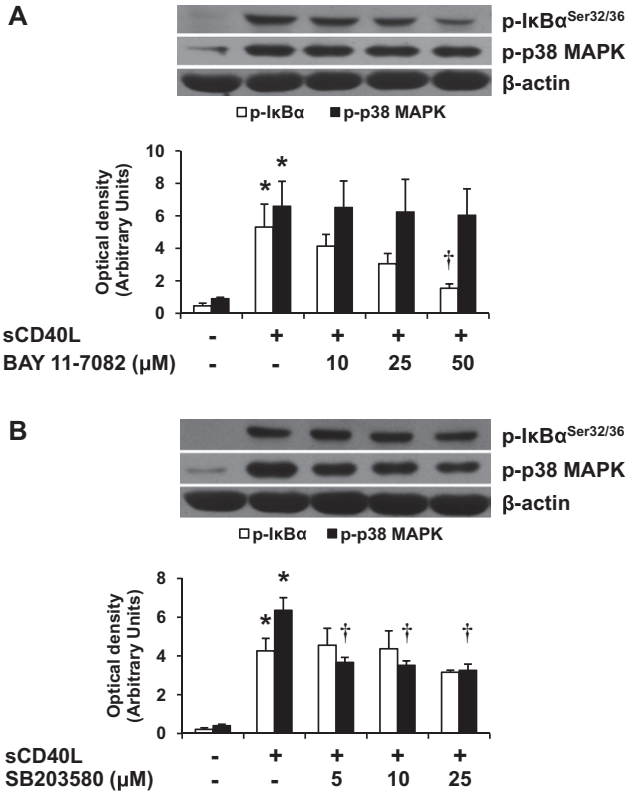

Fig. 2. IκBα phosphorylation downstream of CD40 is independent of p38 MAPK phosphorylation. (A) Dose-dependent effect of BAY 11-7082 on IκBα and p38 MAPK phosphorylation. Platelets (1000×10^6/mL) were incubated with vehicle DMSO or the indicated concentrations of BAY 11-7082 for 5 min at 37 °C and stimulated with sCD40L (1 μg/mL) for 5 min. Platelet lysates where resolved in 12% SDS–PAGE and assessed for p-IκBα Ser$^{32/36}$ ($n = 4$) and phospho-p38 MAPK (p-p38 MAPK) ($n = 3$). (B) Dose-dependent effect of SB203580 on IκBα and p38 MAPK phosphorylation. Platelets were incubated with vehicle DMSO or the indicated concentration of SB203580 for 10 min at 37 °C prior to sCD40L stimulation (1 μg/mL) for 5 min. Platelet lysates where assessed for p-IκBα Ser$^{32/36}$ ($n = 4$) and p-p38 MAPK ($n = 4$). β-Actin blots are from stripped membranes of either p-IκBα or p-p38 MAPK blots. Blots are representative of the indicated number of independent experiments. Histograms represent the mean of data of overlay blots, expressed as arbitrary units of optical density ($^*P < 0.05$ vs. baseline and $^\dagger P < 0.05$ vs. sCD40L alone).

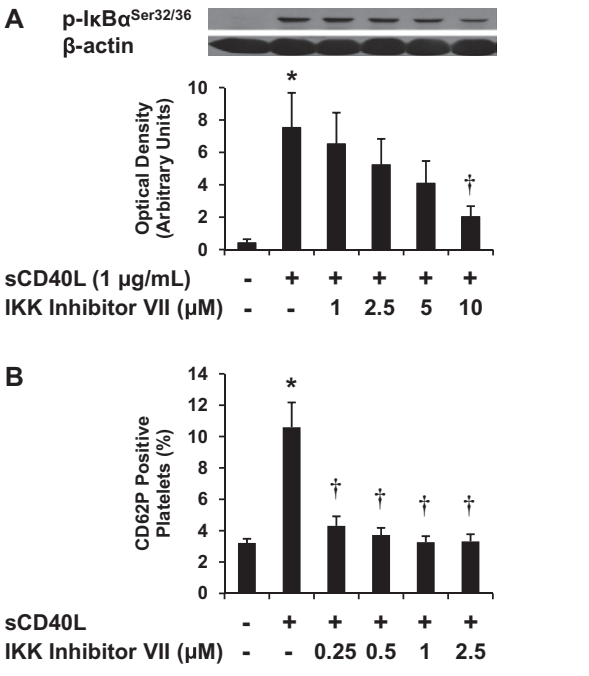

Fig. 3. IκBα is required for sCD40L-induced platelet activation. (A) Platelets (1000×10^6/mL) were incubated with vehicle DMSO or the indicated concentration of IKK inhibitor VII for 5 min at 37 °C and stimulated with sCD40L for 5 min. Platelet lysates where resolved in 12% SDS–PAGE and assessed for p-IκBα Ser$^{32/36}$. β-Actin blot is from stripped membranes of p-IκBα blot. Blots are representative of four independent experiments. Histogram represent the mean of data of overlay blots, expressed as arbitrary units of optical density ($n = 4$; $^*P < 0.05$ vs. baseline and $^\dagger P < 0.05$ vs. sCD40L alone). (B) Effect of IκBα inhibition on platelet activation in response to sCD40L. Platelets (250×10^6/mL) were left untreated or stimulated with sCD40L (1 μg/mL) in the absence or presence of the indicated concentration of IKK inhibitor VII. Platelet activation was assessed by flow cytometry for the expression of CD62P. Histogram represents the mean of data expressed as percent of CD62P positive platelets ($n = 3$; $^*P < 0.05$ vs. baseline and $^\dagger P < 0.05$ vs. sCD40L alone).

3. Results

3.1. sCD40L induces TRAF2 association to CD40 and IκBα phosphorylation in platelets

We first confirmed our previous finding that sCD40L induces the association of TRAF2 to platelet CD40 [19] and showed that pretreatment with a blocking anti-CD40L antibody entirely prevents TRAF2 binding to CD40 in response to sCD40L (Fig. 1A). We then evaluated the activation of the NF-κB signaling pathway downstream of platelet CD40 by assessing IκBα phosphorylation on residues $Ser^{32/36}$, which leads to degradation and release of the active form of NF-κB. Our results show for the first time that sCD40L induces a time-dependent phosphorylation of IκBα in human platelets (Fig. 1B). Since the phosphorylation of IκBα at 5 min was close to maximum, we chose this time point for subsequent experiments. Pretreatment with a blocking anti-CD40L antibody prevents platelet IκBα phosphorylation in response to sCD40L (Fig. 1C).

3.2. IκBα phosphorylation downstream of CD40 is independent of p38 MAPK

Signaling pathways downstream of CD40 have been shown to involve both NF-κB and p38 MAPK [20,21]. Indeed, we have previously shown that sCD40L enhances platelet function through activation of p38 MAPK [19], albeit the link between this MAPK and NF-κB activation remains uncharacterized. In order to delineate the cross-talk between these signaling cascades downstream of platelet CD40, we employed specific inhibitors of IκBα and p38 MAPK phosphorylation, BAY 11-7082 and SB203580 respectively. In platelets, inhibition of IκBα phosphorylation does not influence p38 MAPK phosphorylation following sCD40L treatment, and vice versa (Fig. 2A and B). These results indicate that platelet CD40 signals via two distinct pathways, one involving the NF-κB pathway and another involving p38 MAPK.

3.3. IκBα is required for sCD40L-induced platelet activation and potentiation of aggregation

We have previously shown that sCD40L induces platelet CD62P expression and potentiates platelet aggregation in response to subthreshold doses of platelet agonists [19]. In order to show the importance of IκBα in this process, the IKK inhibitor VII, which targets the kinase responsible for IκBα phosphorylation, was employed. Pretreatment of platelets with the IKK inhibitor VII not only decreases IκBα phosphorylation in a dose-dependent manner (Fig. 3A), but also abolishes platelet activation in response to sCD40L stimulation, as assessed by CD62P expression (Fig. 3B). In functional studies, pretreatment of platelets with either BAY 11-7082 or IKK inhibitor VII reverses the pro-aggregating effects of sCD40L (Fig. 4A and B). These results indicate that the NF-κB signaling pathway, which involves both IκBα and IKK, plays an

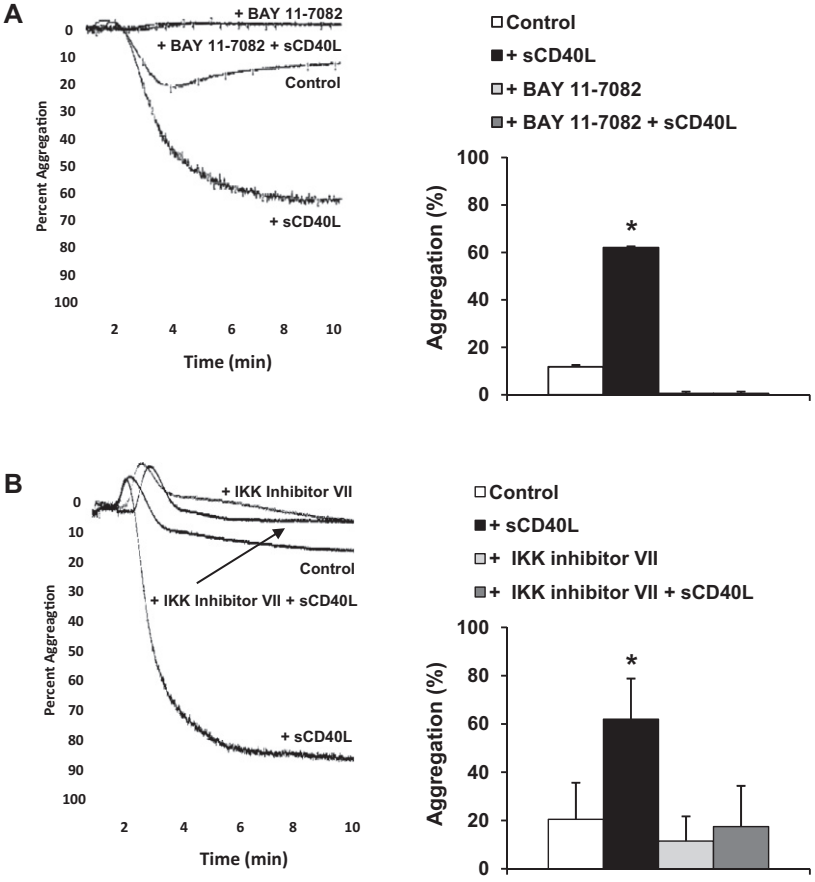

Fig. 4. IκBα is required for sCD40L-induced potentiation of platelet aggregation. (A and B) Effect of IκBα inhibition on the potentiation of platelet aggregation induced by sCD40L. Platelets were preincubated with the IκBα phosphorylation inhibitors BAY 11-7082 (10 μM) or IKK inhibitor VII (2.5 μM), or vehicle DMSO for 5 min at 37 °C. Cells were then left unstimulated (control) or treated with sCD40L (1 μg/mL). Aggregation was then monitor in the presence of a priming dose of collagen (0.5 μg/mL). Histograms represent the mean of data of aggregation traces ($n = 3$; $^{*}P < 0.05$ vs. control).

important role in platelet CD40 signaling, activation and aggregation in response to sCD40L.

4. Discussion

The presence of CD40L in platelets, which is cleaved to generate most of the sCD40L within the circulation, and its ability to induce an inflammatory response in the vascular system is well documented [5,14]. However, its modulation of platelet function remains a matter of debate. Andre et al. have shown that sCD40L binds to $\alpha_{IIb}\beta_3$ and stabilizes arterial thrombi in mice [22]. In contrast, we and others have shown that sCD40L induces platelet activation through CD40 [19,23–25]. Indeed, we have previously demonstrated that sCD40L exacerbates platelet activation and aggregation through a CD40-dependent TRAF2/Rac1/p38 MAPK signaling pathway. We have also shown that elevated levels of sCD40L predisposed platelets to enhanced thrombus formation in response to vascular injury [19]. In this study, we further investigated the signaling pathways downstream of platelet CD40 in response to sCD40L.

In B lymphocytes, it is well documented that CD40 induces activation of the NF-κB signaling pathway through either TRAF2 or TRAF6 [26]. Having shown that sCD40L induces the association of TRAF2/CD40, but not TRAF6/CD40 [19] in platelets, we sought to evaluate whether CD40 ligation could trigger activation of the NF-κB signaling pathway in platelets. We showed for the first time that treatment of platelets with sCD40L activates the NF-κB signaling pathway, which was revealed by the phosphorylation of IκBα on $Ser^{32/36}$. These results add insights to previous work [11], showing that NF-κB may be a novel mediator of platelet responses. Although previous work has focused on the involvement of p38 MAPK in platelet CD40 signaling [19,23], the interrelation between this pathway and the NF-kB cascade remains undetermined. Craxton et al. [27] have shown that p38 MAPK regulates NF-κB activation in B cells, whereas p38 MAPK inhibition does not affect CD40-mediated NF-κB DNA binding, suggesting that NF-κB is not a direct target of the p38 MAPK pathway. In the present study, we show a divergence between the NF-κB and p38 MAPK signaling pathways downstream of platelet CD40. This branching phenomenon suggests that these pathways regulate different aspects of CD40-mediated platelet responses. For instance, the p38 MAPK pathway may be involved in actin polymerization, cytoskeleton reorganization and platelet spreading [28], while the NF-κB pathway may regulate de novo protein synthesis through its interaction with microRNAs [29], which have been shown to be present in platelets [30]. However, further investigations are needed to specifically address this issue.

Malaver et al. [11] have demonstrated the implication of NF-κB in platelet function and showed that blockade of the NF-κB pathway by BAY 11-7082 reverses platelet activation as well as aggregation triggered by thrombin, collagen and ADP. Since we have previously demonstrated that sCD40L induces platelet activation as assessed by α-granule secretion [19]; here we show that pretreatment of platelets with the IKK inhibitor VII, which inhibited IκBα phosphorylation, reversed sCD40L-induced platelet α-granule secretion, as assessed by CD62P translocation to the platelet membrane. Therefore, aside from activating NF-κB, IKK could regulate the activation of critical elements involved in the degranulation process, such as the synaptosomal-associated protein 23 (SNAP-23). In fact, IKK2 regulates mast cell degranulation by phosphorylating SNAP-23 in a NF-κB-independent manner [31]. In functional studies of platelet aggregation, sCD40L stimulation of platelets potentiates aggregation by a mechanism involving the NF-κB signaling pathway, since inhibition of this pathway by either BAY 11–7082 or the IKK inhibitor VII abolished sCD40L-induced potentiation of platelet aggregation. These results indicate that sCD40L via NF-κB, as well as p38 MAPK as previously demonstrated [19], primes platelets and predisposes them to enhanced aggregation responses in the presence of thrombotic stimulus.

In summary, this study shows for the first time that sCD40L is an important inducer of the NF-κB signaling pathway activation in platelets independently of the p38 MAPK pathway. This translates into platelet priming and enhancement of platelet activation and aggregation. Thus, the CD40L/CD40/NF-κB axis may ultimately represent a therapeutic target in the treatment of thrombo-inflammatory diseases.

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