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Regulation of platelet responses triggered by Toll-like receptor 2 and 4 ligands is another non-genomic role of nuclear factor-kappaB

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ABSTRACT

Introduction: Platelets express Toll-like receptors (TLRs) that recognise molecular components of pathogens and, in nucleated cells, elicit immune responses through nuclear factor-kappaB (NF-κB) activation. We have shown that NF-κB mediates platelet activation in response to classical agonists, suggesting that this transcription factor exerts non-genomic functions in platelets. The aim of this study was to determine whether NF-κB activation is a downstream signal involved in TLR2 and 4-mediated platelet responses.
Material and methods: Aggregation and ATP release were measured with a Lumi-aggregometer. Fibrinogen binding, P-selectin and CD40 ligand (CD40L) levels and platelet-neutrophil aggregates were measured by cytometry. I kappa B alpha (IκBα) degradation and p65 phosphorylation were determined by Western blot and von Willebrand factor (vWF) by ELISA.
Results: Platelet stimulation with Pam3CSK4 or LPS resulted in IκBα degradation and p65 phosphorylation. These responses were suppressed by TLR2 and 4 blocking and synergised by thrombin. Aggregation, fibrinogen binding and ATP and vWF release were triggered by Pam3CSK4. LPS did not induce platelet responses *per se*, except for vWF release, but it did potentiate thrombin-induced aggregation, fibrinogen binding and ATP secretion. Pam3CSK4, but not LPS, induced P-selectin and CD40L expression and mixed aggregate formation. All of these responses, except for CD40L expression, were inhibited in platelets treated with the NF-κB inhibitors BAY 11-7082 or Ro 106-9920.
Conclusion: TLR2 and 4 agonists trigger platelet activation responses through NF-κB. These data show another non-genomic function of NF-κB in platelets and highlight this molecule as a potential target to prevent platelet activation in inflammatory or infectious diseases.

Introduction

Platelets are not only critical to haemostatic and thrombotic processes but are also relevant participants in host inflammatory and innate immune responses. In this context, activated platelets express cell adhesion molecules that promote adhesive interactions with leukocytes and endothelial cells, resulting in reciprocal cross-talk that leads to several proinflammatory events, including leukocyte rolling and activation, the production of cytokine cascades, and leukocyte recruitment to sites of tissue damage [1,2].

Among the different mechanisms and molecules involved in inflammation, activation of the nuclear factor-kappa B (NF-κB) has a major role, as it regulates the transcription of several proinflammatory cytokines. Although platelets are anucleated cells, they express functional transcription factors, as the retinoic X receptor [3], peroxisome proliferator-activated receptors γ and β/δ [4–7] and signal transducer and activator of transcription 3 [8]. The activation of these receptors results in both negative and positive regulation of platelet activation.

In 2002, Liu et al. showed that platelets express NF-κB and that thrombin-induced platelet activation triggers the degradation of I kappa B alpha (IκBα), an NF-κB inhibitor [9]. More recently, we reported that NF-κB activation is another signalling pathway involved in classical agonist-mediated platelet activation [10]. These findings were confirmed and further extended by other groups [11–17]. Interestingly,

Abbreviations: Abs, Antibodies; ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; BAY 11-7082, (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile; Ca^{2+}, Calcium; CD40L, CD40 ligand; cGMP/PKG, Cyclic guanosine monophosphate/Dependent protein kinase G; ERK1/2, Extracellular signal-regulated kinase 1/2; FBS, Foetal bovine serum; IκBα, I kappa B alpha; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinase; NF-κB, Nuclear factor-kappa B; Pam3CSK4, Pam3CysSerLys4; PAMPs, Pathogen-associated molecular patterns; PAR, Protease activated receptor; PBS, Phosphate buffered saline; PFA, Paraformaldehyde; PI3-K, Phosphoinositide-3-kinase; PRP, Platelet rich plasma; Ro 106-9920, 6-(phenylsulfinyl)tetrazolo[1,5-b]pyridazine; RT, Room temperature; TLR, Toll-like receptor; TXA_2, Thromboxane A_2; vWF, von Willebrand factor; WPs, Washed platelets.

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IκBα kinase activity appears to be critical to the regulation of platelet secretion, as it induces SNAP-23 phosphorylation, an event required to facilitate the fusion of granule and plasma membranes for cargo release [15]. Additionally, it was recently shown that the signalling transduction pathway involved in thrombin-mediated activation of protease activated receptor (PAR)-4, but not the PAR-1, includes the activation of a neutral sphingomyelinase that increases the intracellular ceramide levels, followed by the activation of a downstream p38 mitogen-activated protein kinase (MAPK)-NF-κB pathway [16]. In contrast to these findings, it was also observed that the NF-κB pathway could play a negative feedback regulatory role in platelet activation [17,18]. Therefore, although these data indicate that NF-κB pathway activation is increasingly recognised as a new signalling pathway in the regulation of platelet biology, the underlying mechanisms of NF-κB function still remain to be established.

The involvement of platelets in innate immune responses is partly due to the expression of Toll-luκe receptors (TLRs). These receptors detect and recognise pathogen-associated molecular patterns (PAMPs) that are not present in host cells. Each TLR responds to a different set of ligands or PAMPs from bacteria, viruses and fungi. Although platelets express several TLR family members [19,20], TLR2 and 4 have been the most studied because they recognise PAMPs present in Gram-positive and negative bacteria, respectively [21], and because platelets not only significantly contribute to the pathophysiology of sepsis but are also involved in the high mortality levels associated with this disease [22–24].

TLR2 forms heterodimers with TLR1 or TLR6, and these are essential to the identification of various microbial cell wall components, including lipoproteins and peptidoglycans [20]. Although an early study reported that TLR2 agonists were unable to modulate ADP or platelet activating factor-mediated platelet activation [25], other groups demonstrated that the triacetylated lipopeptide Pam3Cys-Ser-(Lys)4 (Pam3CSK4) can activate platelet haemostatic and inflammatory responses through binding to the TLR2/1 complex [26–29]. This activation was associated with increased phosphoinositide-3-kinase (PI3-K) activity and the phosphorylation of Akt, extracellular signal-regulated kinase (ERK)1/2 and p38, as well as P2X1-mediated Ca^{2+} mobilisation, TXA_2 production and ADP receptor activation [26–28,30]. Data regarding TLR4 activation are more controversial. More specifically, platelet activation mediated by lipopolysaccharide (LPS), a component of all Gram-negative bacteria and a TLR4 agonist, has been reported to be incapable of inducing platelet responses [25], to promote platelet activation [31–33], to enhance classical agonist-induced platelet aggregation [31,34], and to inhibit platelet activation [35].

Because NF-κB is a downstream signal of the TLRs in nucleated cells [21] and its participation in TLR2 or 4 agonist-stimulated platelets is not yet known, in this study we explored whether NF-κB is involved in platelet responses mediated by these immune receptors.

Materials and Methods

Reagents

Human α-thrombin was purchased from Enzyme Research Labs (Swansea, UK). Purified LPS derived from *E. coli* O111:B4, luciferin-luciferase and ATP were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pam3CSK4 was purchased from InvivoGen (San Diego, CA, USA). Alexa 488-fibrinogen was obtained from Invitrogen (Eugene, OR, USA). FITC-conjugated anti-human CD62P, FITC-anti-CD45, FITC-anti-CD40L, PE-anti-CD61, irrelevant FITC-or PE IgG1 and unlabelled IgG1, mouse anti-IκBα and anti-β-actin were obtained from BD Biosciences (San José, CA, USA). Mouse anti-phospho NF-κB p65 (Ser311), total NF-κB p65 and HRP-conjugated anti-rabbit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blocking antibodies (Abs) against human TLR2 (clone T2.5) and TLR4 (HTA125) were obtained from eBioscience (San Diego, CA, USA). The NF-κB inhibitors (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile (BAY 11-7082) and 6-(phenylsulfinyl)tetrazolo[1,5-b]pyridazine (Ro 106-9920) were purchased from Biomol (Plymouth Meeting, PA, USA) and Tocris (Ellisville, MO, USA), respectively. These drugs were dissolved in DMSO. The final DMSO concentration (0.5% v/v) did not have a toxic effect. Where indicated, platelets were depleted from leukocytes by using a high efficiency leukoreduction filter (Purecell PL, PALLBiomedical Products, East Hills, NY, USA).

Preparation of Human Platelets

Blood samples were obtained from healthy donors who had not taken any non-steroidal anti-inflammatory drugs for 10 days prior to sampling. This study was performed according to institutional guidelines and was approved by the Institutional Ethics Committee. Written consent was obtained from all of the subjects. Platelet-rich plasma (PRP) was obtained by blood sample centrifugation. To avoid leukocyte contamination only the top 75% of the PRP was collected. For washed platelet (WP) suspensions, PRP was centrifuged in the presence of 75 nM prostacyclin (PGI_2, Cayman, Ann Arbor, MI, USA). After washing, the WPs were resuspended in Tyrode's buffer, kept at room temperature (RT) for 30 min prior to experimentation, and Ca^{2+} (1 mM) was added prior to platelet stimulation. WPs were stimulated for 15 min with different concentrations of Pam3CSK4 or LPS that were prepared in buffer with foetal bovine serum (FBS, 1%) to provide the necessary cofactors for its action, or with thrombin that was prepared in buffer without serum. All experiments were performed at 37 °C.

Immunoblotting

WPs were stimulated with Pam3CSK4, LPS or thrombin and then solubilised in loading buffer (62.5 mM Tris-HCl at pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol). Equal amounts of sample proteins were electrophoresed on a 10% SDS-PAGE gel and electrotransferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary Abs, followed by incubation with HRP-conjugated secondary Abs. Protein bands were visualised by ECL. Immunoblotting results were quantified using the integrated optical density (IOD) of each band with Gel-Pro Analyzer 3.1 software, and the values from blot reprobes were used to monitor equal protein loads.

Platelet Aggregation and ATP Release

Aggregation and ATP release were measured simultaneously in a Lumiaggregometer (Chrono-Log, Havertown, PA, USA). ATP levels were calculated at the end of the assay by adding a known amount of ATP. The aggregation results were expressed as the percentage of maximal light transmission.

Flow Cytometry Analysis

WPs were stimulated, fixed and stained with FITC-CD62P (anti-P-selectin) or FITC-CD40L or an equivalent amount of isotype-matched control Ab in PBS with 0.1% FBS. In selected experiments, WPs were pre-treated with the inhibitors BAY 11-7082 (25 μM) and Ro 106-9920 (12.5 μM) for 5 min. To measure fibrinogen binding, WPs were stimulated in the presence of Alexa-488 fibrinogen. Samples were analysed by flow cytometry on a FACSCalibur using CELLQUEST software (BD Biosciences, Franklin Lakes, NJ, USA) and the results were expressed as percentage of positive cells or mean of fluorescence intensity (MFI).

Measurement of von Willebrand Factor (vWF)

WPs were stimulated, with either Pam3CSK4 or LPS. The reactions were halted by the addition of PBS with 75 nM PGI_2. The samples

were centrifuged first at 1100 x *g* for 5 min, followed by 9300 x *g* for 5 min and the supernatants were stored at -80 °C until assayed. vWF release was determined by ELISA as previously described [36].

Platelet-neutrophil Mixed Aggregates Assays

Human neutrophils were prepared by Ficoll-Hypaque gradient centrifugation and dextran sedimentation as previously described [36]. Equal volumes of WPs ($2x10^8$/ml) and neutrophils ($5x10^6$/ml) were incubated together in Tyrode's buffer for 20 min, followed by stimulation with either Thr (0.01 U/ml), Pam3CSK4 (1 μg/ml) or LPS (1 μg/ml) for 15 min. When it was indicated, WPs were pretreated with the inhibitors BAY 11-7082 (25 μM) and Ro 106-9920 (12.5 μM) for 5 min. After stimulation, the samples were fixed with 1% PFA, stained with FITC-CD45 and PE-CD61 Abs or equivalent amounts of FITC and PE-isotype-matched controls and then analysed by flow cytometry. The results were expressed as the percentage of $CD45^+/CD61^+$ events within the total $CD45^+$ population.

Statistical Analysis

The data are expressed as the means ± SEM. ANOVA and the Newman-Keuls test were employed to determine the significant differences between the groups. A p value less than 0.05 was considered to be statistically significant.

Results

Platelet TLR2 and 4 Activation Results in Downstream Stimulation of the NF-κB Pathway

To determine whether activated NF-κB is a downstream molecule involved in TLR2 and 4 signalling in platelets, the cells were stimulated with Pam3CSK4 or LPS. Western blot studies showed that the activation of both receptors resulted in the degradation of the NF-κB inhibitor, IκBα, and the phosphorylation of the NF-κB p65 subunit. While both events were completely reversed by platelet pretreatment with Abs against human TLR2 and 4 (Fig. 1A and B), a control IgG had no effect (data not shown), indicating that NF-κB activation was specifically associated with the TLR.

Because NF-κB activation is triggered upon thrombin-mediated platelet stimulation [10] and the coagulation cascade is usually activated during infection, we next investigated the effect of thrombin in combination with Pam3CSK4 or LPS. Fig. 2A and B show that thrombin-triggered IκBα degradation and NF-κB p65 subunit phosphorylation were significantly increased by different concentrations

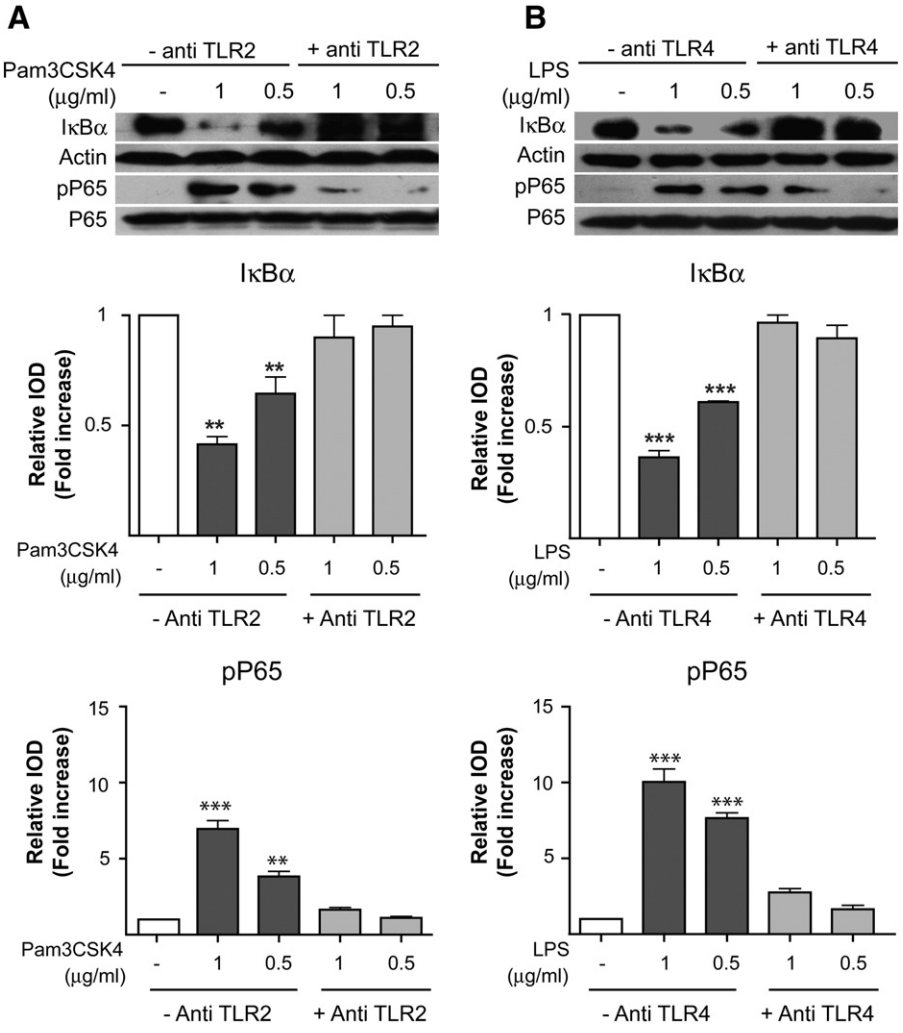

Fig. 1. The NF-κB pathway in platelets is activated by TLR2 and TLR4 agonists. WPs ($1x10^9$/ml) were stimulated at 37 °C with (A) Pam3CSK4 or (B) LPS for 15 min. When indicated, the cells were preincubated for 20 min with TLR2 (50 μg/ml) or TLR4 (50 μg/ml) blocking Abs. The levels of IκBα degradation and p65 phosphorylation were determined in cell lysates. Each membrane was reprobed with anti-β-actin or total p65 antibodies respectively to calculate the relative IOD. The images are representative of three independent experiments (** $p < 0.01$, *** $p < 0.001$ vs. the unstimulated control).

of Pam3CSK4 or LPS, indicating synergism between thrombin and TLR2 or 4 activation.

NF-κB Activation is Necessary for Pam3CSK4 and LPS-mediated Aggregation and Fibrinogen Binding

To evaluate the functional role of NF-κB in Pam3CSK4 and LPS-mediated platelet activation, we next examined several platelet responses triggered by these TLR agonists in the presence of BAY 11-7082 or Ro 106-9920, two specific and unrelated inhibitors of NF-κB activation [10]. Initially, we analysed platelet aggregation. In agreement with previous reports [26,28], we found that platelet stimulation with Pam3CSK4 promoted platelet aggregation in a concentration-dependent manner ($EC_{50} = 5 \pm 0.2$ μg/ml, $n = 3$). This response was observed with or without the addition of serum ($n = 3$, data not shown). In contrast, LPS was not able to trigger aggregation but did potentiate the response mediated by thrombin (Fig. 3A). Moreover, the presence of serum was required to observe the effects of LPS on platelet activation (data not shown). Preincubation of the platelets with both NF-κB inhibitors completely suppressed the aggregatory effects mediated by TLR2 and 4 stimulation (Fig. 3A).

To further understand the molecular mechanisms involved in this effect and because platelet aggregation is mediated by fibrinogen binding, we next evaluated this process in the absence or presence of BAY 11-7082 or Ro 106-9920. Like platelet aggregation, fibrinogen binding was directly induced by Pam3CSK4; in the case of LPS, binding was triggered only when this molecule was used in combination with thrombin (Fig. 3B). These responses were significantly decreased in platelets that had been pretreated with the NF-κB inhibitors (Fig. 3B).

Because it was previously reported that LPS-mediated platelet responses are associated with the activation of contaminating leukocytes in platelet suspensions [37], the platelets were further purified with a high-efficiency leukoreduction filter to rule out this possibility. Flow cytometry analysis showed that $CD45^+$ and $CD14^+$ cells were not detected in filtered platelet suspensions. Under these conditions, the LPS priming of thrombin-mediated aggregation and fibrinogen binding was similar to that observed in non-filtered platelets (data not shown).

Inhibition of NF-κB Impaired Pam3CSK4 and LPS-induced Platelet Granule Release

Because previous studies reported that both Pam3CSK4 [26] and LPS [31] could induce the release of granule contents, we examined whether NF-κB participates in platelet granule release triggered by the stimulation of both TLRs. For this purpose, ATP and vWF secretion were determined as markers of dense and α granules release, respectively. As observed with platelet aggregation, Pam3CSK4 promoted ATP release,

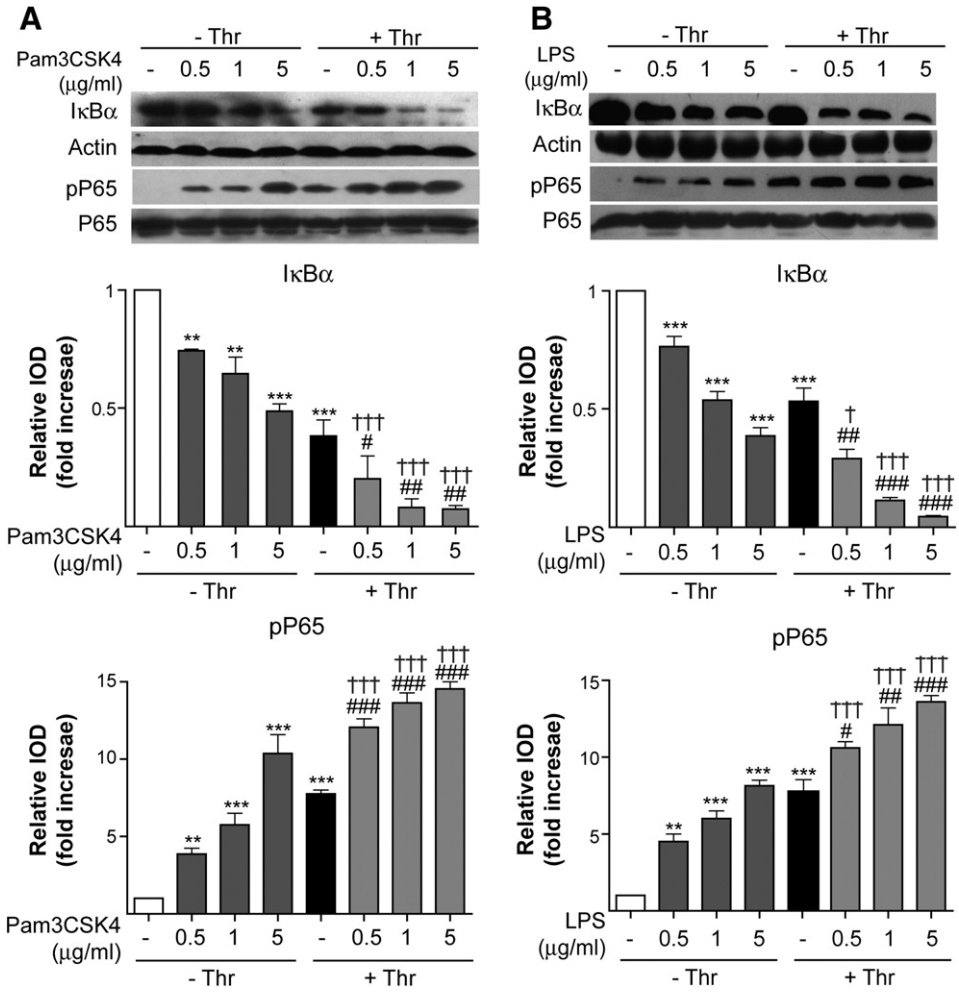

Fig. 2. TLR2 and TLR4 agonists enhance thrombin-triggered NF-κB activation. WPs ($1x10^9$/ml) were stimulated at 37 °C with (A) Pam3CSK4 or (B) LPS for 15 min with or without thrombin (Thr) (0.01 U/ml). The levels of IκBα degradation and p65 phosphorylation were determined in cell lysates. Each membrane was reprobed with anti-β-actin or total p65 antibodies respectively to calculate the relative IOD. The images are representative of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the unstimulated control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Thr alone; † $p < 0.05$, ††† $p < 0.001$ vs. the same concentration of Pam3CSK4 or LPS alone).

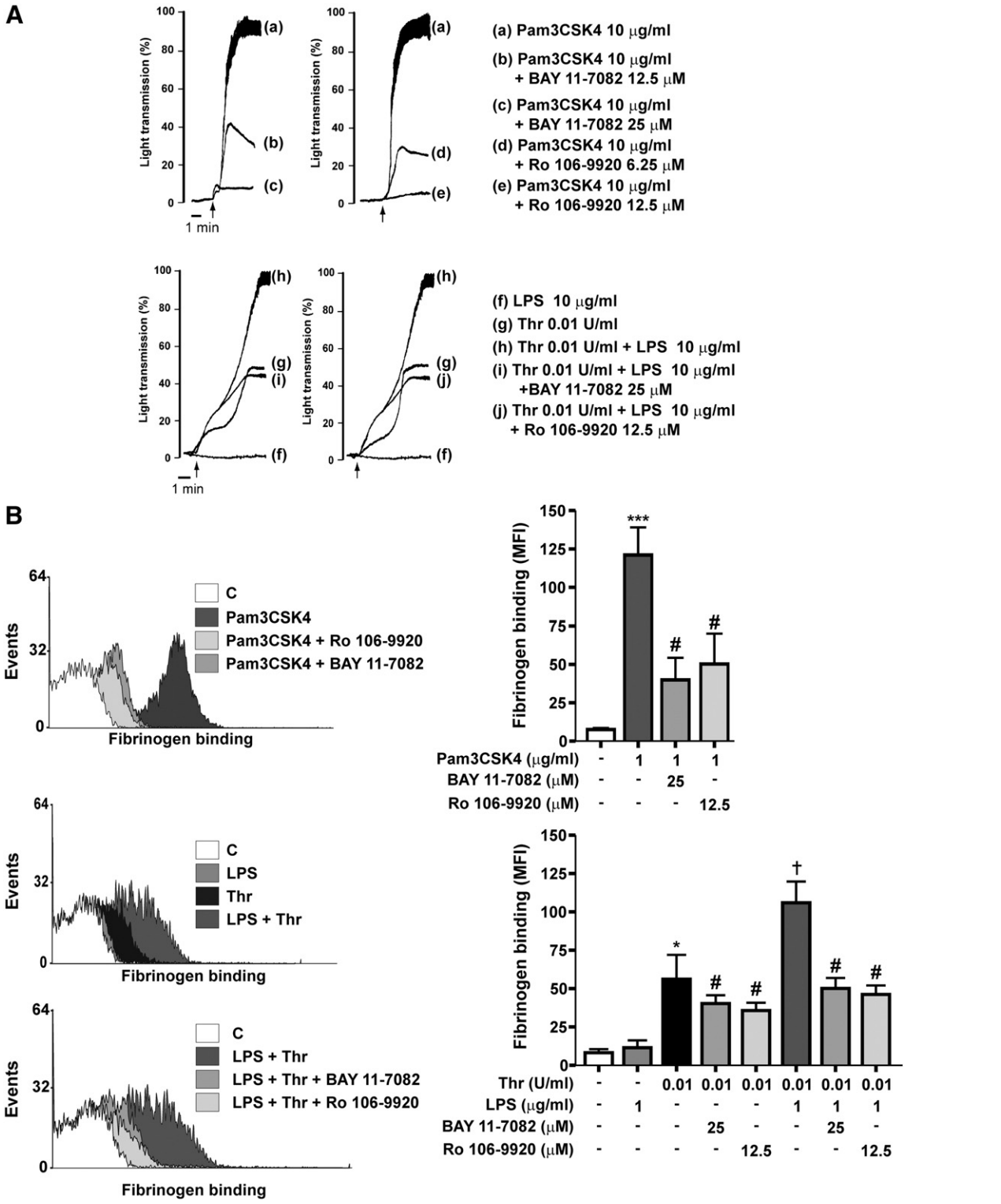

Fig. 3. Pam3CSK4 and LPS-mediated platelet aggregation and fibrinogen binding requires NF-κB activation. WPs ($3x10^8$/ml) were stimulated with Pam3CSK4 or Thr and LPS in the presence or absence of BAY 11-7082 or Ro 106-9920 and (A) platelet aggregation or (B) fibrinogen binding were measured (n = 3, * $p < 0.05$, *** $p < 0.001$ vs. the unstimulated control; # $p < 0.05$ vs. Pam3CSK4, Thr or Thr and LPS; † $p < 0.05$ vs. Thr).

whereas LPS alone did not; however, LPS potentiated this response in combination with thrombin (Fig. 4A). In contrast, platelet stimulation with either TLR agonist resulted in a modest release of vWF that was statistically significant (Fig. 4B).

Both ATP or vWF secretion were suppressed in the presence of BAY 11-7082 or Ro 106-9920, supporting the concept that NF-κB is a mediator in both dense and α granules content release in response to TLR2 and 4 ligands (Fig. 4A and B).

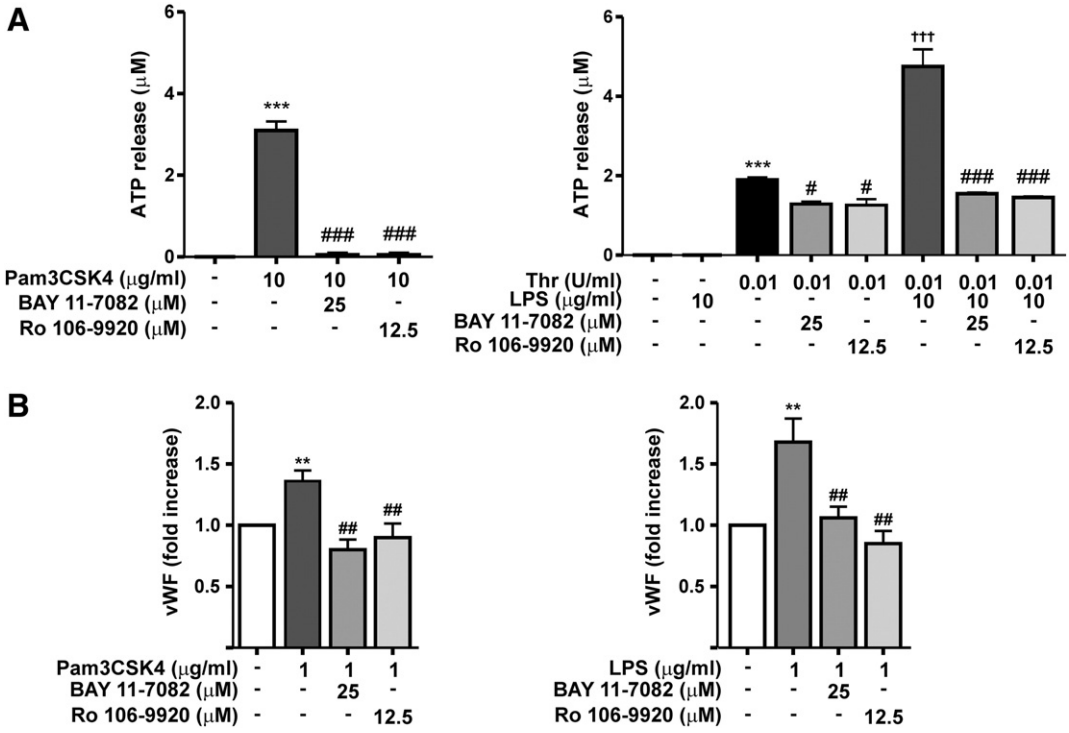

Fig. 4. Pam3CSK4 and LPS trigger platelet granule release via NF-κB activation. WPs ($3x10^8$/ml) were stimulated with Pam3CSK4, LPS or Thr and LPS in the presence or absence of BAY 11-7082 or Ro 106-9920. (A) ATP or (B) vWF release were measured ($n = 5$, ** $p < 0.01$, *** $p < 0.001$ vs. the unstimulated control; #$p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. Pam3CSK4, Thr alone, Thr and LPS or LPS alone; ††† $p < 0.001$ vs. Thr and LPS).

Pam3CSK4 but not LPS Induces NF-κB-mediated Proinflammatory Responses

Having observed that NF-κB mediated the haemostatic platelet responses induced by TLR2 or 4 stimulation, we then examined its role in primary platelet-mediated proinflammatory responses, including the expression of cell adhesion molecules such as CD40L, P-selectin and platelet-leukocyte aggregates.

Flow cytometry studies revealed that platelet stimulation with Pam3CSK4 triggered CD40L expression on the platelet surface, but this expression was not modified by treatment of platelets with NF-κB inhibitors. LPS neither induced CD40L expression nor modified the thrombin-induced expression (Fig. 5A).

Platelet stimulation with Pam3CSK4 also induced P-selectin expression (Fig. 5B) and the formation of platelet-leukocyte aggregates (Fig. 5C), and both platelet responses were significantly reduced in platelets that had been pretreated with BAY 11-7082 or Ro 106-9920. Again, LPS could not induce either response alone and did not modify thrombin-mediated P-selection expression or mixed aggregates formation (Fig. 5B and C).

Discussion

We previously demonstrated that NF-κB is expressed in platelets and that platelet pretreatment with NF-κB blocking agents markedly reduces platelet function, suggesting that NF-κB activation is another pathway involved in the generation of platelet responses [10]. We now show that NF-κB is not only a mediator of classical agonist-triggered platelet activation but is also a critical downstream signal involved in TLR2 and 4 stimulation-induced platelet effector responses. Our data demonstrate that the activation of TLR2 and 4 by their respective ligands, Pam3CSK4 and LPS, resulted in both IκBα degradation and NF-κB p65 subunit phosphorylation, two events that are intimately associated with NF-κB activation. This effect was specifically linked to TLR2 and 4 activation, as both phenomena were potently suppressed by platelet preincubation with neutralising Abs against these TLRs. Interestingly, Pam3CSK4 or LPS synergised with thrombin to trigger full NF-κB activation. The functional relevance of platelet NF-κB activation was demonstrated by the observation that all Pam3CSK4 or LPS-triggered platelet responses, except for CD40L expression, were significantly abrogated when platelets were treated with BAY 11-7082 or Ro 106-9920, two structurally non-related inhibitors of NF-κB activation. In this sense and in agreement with previous studies, we found that platelet stimulation with Pam3CSK4 resulted in concentration-dependent aggregation and dense (ATP) and α (vWF, CD40L and P-selectin) granules release (Fig. 6). In contrast to Pam3CSK4, platelet responses associated with LPS stimulation were more varied. LPS *per se* could not trigger platelet responses beyond NF-κB activation and vWF release. Although we did not observe a direct effect of LPS, platelet aggregation, fibrinogen binding and ATP release in response to threshold thrombin concentrations were primed by the presence of LPS, and this effect was suppressed by blocking the activation of NF-κB, indicating a relevant role of this pathway in the priming effects of LPS (Fig. 6). The different sensitivities of platelet responses to LPS are unclear, but one possibility is that LPS signalling involves molecules that are more directly associated with the regulation of granule secretion than aggregation. Previous studies regarding LPS-mediated platelet activation have been controversial. Some studies demonstrated that LPS-mediated activation triggered dense and α granule content release [31,38], whereas others observed that LPS synergised with platelet agonists [31,34]. Still others reported that LPS regulated platelet function in a negative manner [35] or that it did not directly induce platelet activation [25]. Interestingly, our results demonstrate that LPS promotes differential α granule release by inducing vWF release but not P-selectin expression, thus associating bacterial products with the novel concept of differential platelet secretion [39] for the first time. In nucleated cells, TLR activation triggers a complex intracellular signalling cascade that leads to NF-κB activation and proinflammatory gene induction [21]. Although the signalling pathways that mediate platelet activation by TLR2 stimulation are not yet completely elucidated,

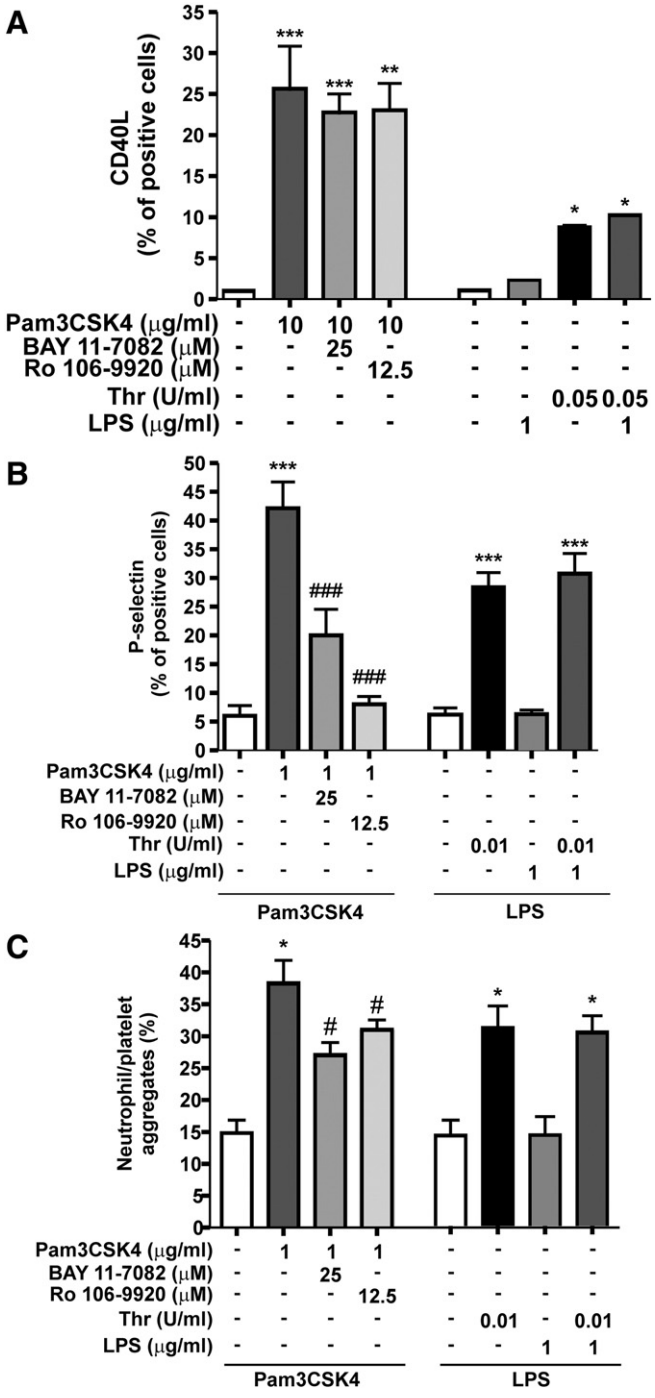

Fig. 5. Pam3CSK4 but not LPS induces NF-κB-mediated proinflammatory responses. WPs ($3x10^7$/ml) were stimulated with Pam3CSK4 or Thr and LPS in the presence or absence of BAY 11-7082 or Ro 106-9920 and (A) CD40L and (B) P-selectin expression were analysed. (C) Equal volumes of platelets ($2x10^8$/ml) and neutrophils ($5x10^6$/ml) were co-incubated for 20 min and then stimulated with either Pam3CSK4 or Thr and LPS 5 min. Results are expressed as the percentage of $CD61^+$ events within the total $CD45^+$ population ($n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the unstimulated control; # $p < 0.05$, ### $p < 0.001$ vs. Pam3CSK4).

activation of the PI3-K/Akt axis, phosphorylation of ERK1/2 and p38, as well as Ca^{2+} mobilisation were shown to be involved [26,28–30]. Studies that have observed LPS-mediated platelet activation have determined that LPS stimulates platelet secretion and potentiates platelet activation through a TLR4/MyD88 and cGMP/PKG-dependent pathway [31]. Our data showing that none of the Pam3CSK4 or LPS-triggered platelet responses were elicited in the presence of the NF-κB inhibitors suggested that activated NF-κB is also an effector molecule necessary for the stimulation of platelet responses through TLR2 and 4 and further strengthens the notion that even in the absence of a nucleus, NF-κB exerts relevant non-genomic functions (Fig. 6).

Platelet activation not only results in haemostatic responses that are critical to normal haemostasis but also induces the expression, synthesis and release of molecules that are important factors in inflammatory responses. Among these, P-selectin and CD40L expression promote interactions between platelets and leukocytes and endothelial cells that are conducive to the reciprocal activation of the three cell types and the amplification of the inflammatory response [1,2]. As in previous reports, we found that platelet activation with Pam3CSK4 triggered the expression of both cell adhesion molecules and the formation of platelet-leukocyte aggregates [26]. Although the treatment of platelets with NF-κB inhibitors significantly reduced P-selectin expression and mixed aggregate formation, the effects of these inhibitors were lower than those exerted on platelet haemostatic responses. Moreover, CD40L expression was not affected by the presence of the inhibitors (Fig. 6). In this context, it is possible that the effector molecules involved in the inflammatory-mediated effects elicited by Pam3CSK4 include the activation of IRAK kinases, which are not influenced by BAY 11-7082 or Ro 106-9920. Consistent with previous data [25] and in contrast to our observations with Pam3CSK4, LPS did not trigger P-selectin or CD40L expression, and LPS-mediated activation of platelets and leukocytes did not result in mixed cell aggregate formation either alone or in combination with thrombin. Unlike the *in vitro* situation, LPS-mediated platelet and neutrophil interactions were demonstrated *in vivo* [23,40]. Moreover, this appears to be a relevant phenomenon in sepsis thrombocytopenia and in the formation of neutrophil extracellular traps. Additionally, platelet TLR4 is a critical molecule that mediates these effects [22]. Thus, further experiments are required to determine whether platelet NF-κB activation is required in the LPS-mediated cross-talk between platelets and neutrophils.

Increasing numbers of *in vitro* and *in vivo* studies suggest that platelet activation in response to bacteria could be a pathogenic mechanism responsible for the frequent association between thrombosis and infection as well as a factor in the development of atherosclerosis [41,42]. Thus, identification of the molecular mechanisms involved becomes relevant for the improvement or development of new therapies.

In this study, we show that NF-κB pathway is a mediator in TLR2 and 4-triggered platelet activation responses. A usual concern about the use of pharmacological inhibitors is their potential off target effects. In this sense, it has been demonstrated that BAY 11-7082 does not act as a global inhibitor of cytokine-mediated phosphorylation, but selectively inhibits the phosphorylation of IκBα [43]. In addition, Ro 106-9920, despite not being structurally related to BAY 11-7082, selectively inhibits IκBα by interfering with its ubiquitination, but not its phosphorylation [44]. In fact, we have previously shown that while BAY 11-7082 inhibited IκBα phosphorylation induced by thrombin in a concentration-dependent manner, Ro 106-9920 had no effect [10], strengthening the notion that the two drugs have different mechanisms of action. Moreover, a recent study using platelet specific IKKβ knockout mice (KO) showed that blocking IKKβ activity significantly prolonged tail-bleeding times giving further support to the novel notion that elements of NF-κB/IκBα cascade are mediators of platelet activation [15]. Thus, although we do not rule out the possibility that BAY 11-7082 and Ro 106-9920 may have alternative modes of action, it is highly conceivable that the inhibitory effect of these compounds on platelet function is due to a selective inhibition of the NF-κB pathway.

In conclusion, our findings present another non-genomic function of this transcription factor and suggest that this molecule could represent a potentially interesting target to control platelet activation in infectious diseases. Further human and animal models with platelet specific receptor KO are required to explain the relevance of our in-vitro findings.

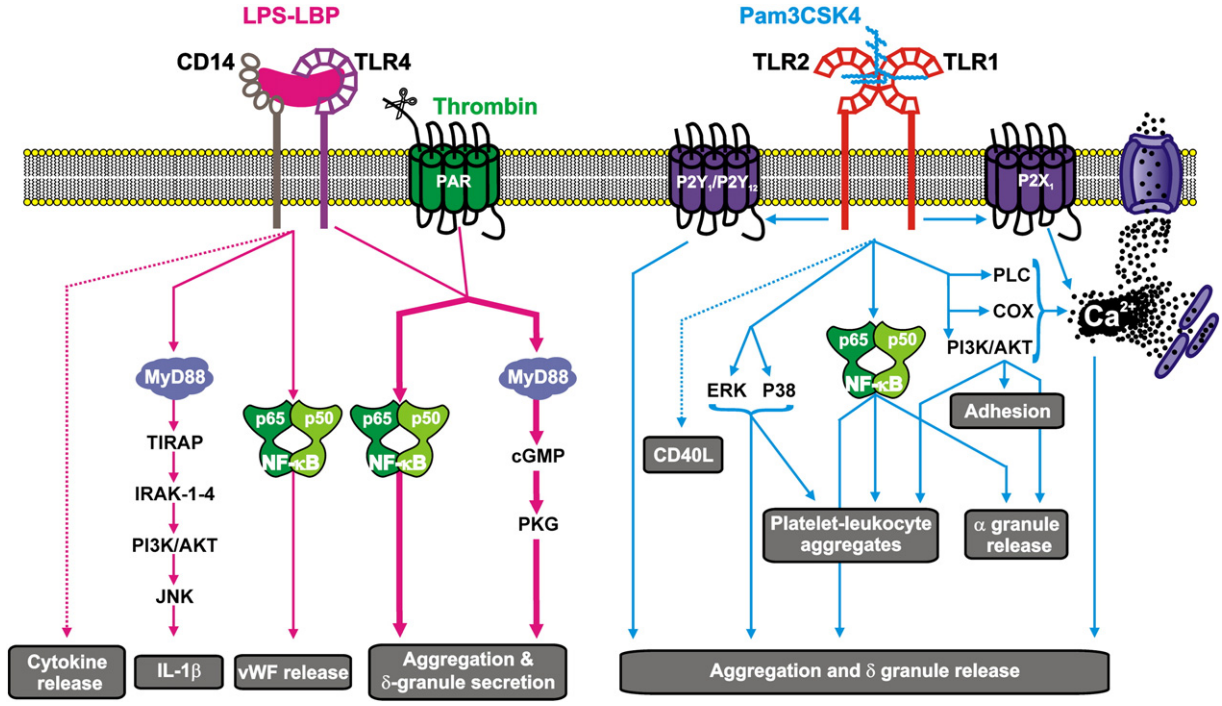

Fig. 6. Mechanisms involved in TLR 2 and 4-mediated platelet activation. The picture represents the principal mechanisms involved in platelet activation mediated by Pam3CSK4 and LPS, TLR2/1 and 4 agonists, respectively. LPS binds to LPS-binding protein (LPB) and CD14 to promote platelet activation via TLR4. LPS directly induces the release of von Willebrand Factor (vWF) through NF-κB and the proinflammatory IL-1-β by activating MyD88-TIRAP, IRAK-1-4, PI3K/AKT and JNK pathway [33]. Also, it potentiates thrombin-mediated platelet aggregation and dense granule secretion via NF-κB- and MyD88/cGMP/PKG-dependent mechanisms (bold line) [31]. It has also been reported that LPS does not induce [25] or inhibit platelet activation [35]. The stimulation of platelets with Pam3CSK4 triggers several platelet responses through interaction with TLR2/1. An ATP-dependent intracellular Ca^{2+} mobilisation, production of TxA_2, purinergic activation of P2Y1 and P2Y12 receptors, the activation of PI3K/AKT pathway [26,28], phosphorylation of MAP kinases p38 and ERK1/2 [30] as well as activation of NF-κB are involved in the formation of platelet/leukocyte aggregates, platelet adhesion, aggregation and dense and alpha granule release. The dashed lines indicate that the mechanisms involved are unknown.

Addendum

L. Rivadeneyra and A. Carestia equally performed experiments, interpreted results and assisted with manuscript preparation; J. Etulain performed ELISA studies and assisted with manuscript editing, RG. Pozner, S. Negrotto, and C. Fondevila analyzed data, interpreted results and assisted with manuscript editing. M. Schattner designed the study, interpreted results, formulated discussion, wrote the manuscript and assisted with manuscript preparation and editing. All authors read and approved the final manuscript.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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References

[1] Jenne CN, Urrutia R, Kubes P. Platelets: bridging hemostasis, inflammation, and immunity. Int J Lab Hematol 2013;35:254–61.
[2] Etulain J, Schattner M. Current viewpoints on platelet contribution to inflammation. World J Hematol 2012;1:14–21.
[3] Moraes LA, Swales KE, Wray JA, Damazo A, Gibbins JM, Warner TD, et al. Nongenomic signaling of the retinoid X receptor through binding and inhibiting Gq in human platelets. Blood 2007;109:3741–4.
[4] Ali FY, Davidson SJ, Moraes LA, Traves SL, Paul-Clark M, Bishop-Bailey D, et al. Role of nuclear receptor signaling in platelets: antithrombotic effects of PPARbeta. FASEB J 2006;20:326–8.
[5] Moraes LA, Spyridon M, Kaiser WJ, Jones CI, Sage T, Atherton RE, et al. Non-genomic effects of PPARgamma ligands: inhibition of GPVI-stimulated platelet activation. J Thromb Haemost 2010;8:577–87.
[6] Ali FY, Hall MG, Desvergne B, Warner TD, Mitchell JA. PPARbeta/delta agonists modulate platelet function via a mechanism involving PPAR receptors and specific association/repression of PKCalpha–brief report. Arterioscler Thromb Vasc Biol 2009;29:1871–3.
[7] Akbiyik F, Ray DM, Gettings KF, Blumberg N, Francis CW, Phipps RP. Human bone marrow megakaryocytes and platelets express PPARgamma, and PPARgamma agonists blunt platelet release of CD40 ligand and thromboxanes. Blood 2004;104:1361–8.
[8] Zhou Z, Gushiken FC, Bolgiano D, Salsbery BJ, Aghakasiri N, Jing N, et al. Signal transducer and activator of transcription 3 (STAT3) regulates collagen-induced platelet aggregation independently of its transcription factor activity. Circulation 2013;127:476–85.
[9] Liu F, Morris S, Epps J, Carroll R. Demonstration of an activation regulated NF-kappaB/I-kappaBalpha complex in human platelets. Thromb Res 2002;106:199–203.
[10] Malaver E, Romaniuk MA, D'Atri LP, Pozner RG, Negrotto S, Benzadon R, et al. NF-kappaB inhibitors impair platelet activation responses. J Thromb Haemost 2009;7:1333–43.
[11] Chang CC, Lu WJ, Ong ET, Chiang CW, Lin SC, Huang SY, et al. A novel role of sesamol in inhibiting NF-kappaB-mediated signaling in platelet activation. J Biomed Sci 2011;18:93.
[12] Hachem A, Yacoub D, Zaid Y, Mourad W, Merhi Y. Involvement of nuclear factor kappaB in platelet CD40 signaling. Biochem Biophys Res Commun 2012;425:58–63.
[13] Spinelli SL, Casey AE, Pollock SJ, Gertz JM, McMillan DH, Narasipura SD, et al. Platelets and megakaryocytes contain functional nuclear factor-kappaB. Arterioscler Thromb Vasc Biol 2010;30:591–8.
[14] Lee HS, Kim SD, Lee WM, Endale M, Kamruzzaman SM, Oh WJ, et al. A noble function of BAY 11-7082: Inhibition of platelet aggregation mediated by an elevated cAMP-induced VASP, and decreased ERK2/JNK1 phosphorylations. Eur J Pharmacol 2010;627:85–91.
[15] Karim ZA, Zhang J, Banerjee M, Chicka MC, Al Hawas R, Hamilton TR, et al. IkappaB kinase (IKK) phosphorylation of SNAP-23 controls platelet secretion. Blood 2013;121:4567–74.
[16] Chen WF, Lee JJ, Chang CC, Lin KH, Wang SH, Sheu JR. Platelet protease-activated receptor (PAR)4, but not PAR1, associated with neutral sphingomyelinase responsible for thrombin-stimulated ceramide-NF-kappaB signaling in human platelets. Haematologica 2013;98:793–801.
[17] Wei S, Wang H, Zhang G, Lu Y, An X, Ren S, et al. Platelet IkappaB kinase-beta deficiency increases mouse arterial neointima formation via delayed glycoprotein Ibalpha shedding. Arterioscler Thromb Vasc Biol 2013;33:241–8.

[18] Gambaryan S, Kobsar A, Rukoyatkina N, Herterich S, Geiger J, Smolenski A, et al. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. J Biol Chem 2010;285:18352–63.
[19] Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C, Garraud O. Evidence of Toll-like receptor molecules on human platelets. Immunol Cell Biol 2005;83:196–8.
[20] Beaulieu LM, Freedman JE. The role of inflammation in regulating platelet production and function: Toll-like receptors in platelets and megakaryocytes. Thromb Res 2010;125:205–9.
[21] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010;11:373–84.
[22] Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med 2007;13:463–9.
[23] Aslam R, Speck ER, Kim M, Crow AR, Bang KW, Nestel FP, et al. Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production in vivo. Blood 2006;107:637–41.
[24] Li Z, Yang F, Dunn S, Gross AK, Smyth SS. Platelets as immune mediators: their role in host defense responses and sepsis. Thromb Res 2011;127:184–8.
[25] Ward JR, Bingle L, Judge HM, Brown SB, Storey RF, Whyte MK, et al. Agonists of toll-like receptor (TLR)2 and TLR4 are unable to modulate platelet activation by adenosine diphosphate and platelet activating factor. Thromb Haemost 2005;94:831–8.
[26] Blair P, Rex S, Vitseva O, Beaulieu L, Tanriverdi K, Chakrabarti S, et al. Stimulation of Toll-like receptor 2 in human platelets induces a thromboinflammatory response through activation of phosphoinositide 3-kinase. Circ Res 2009;104:346–54.
[27] Fung CY, Jones S, Ntrakwah A, Naseem KM, Farndale RW, Mahaut-Smith MP. Platelet Ca(2+) responses coupled to glycoprotein VI and Toll-like receptors persist in the presence of endothelial-derived inhibitors: roles for secondary activation of P2X1 receptors and release from intracellular Ca(2+) stores. Blood 2012;119:3613–21.
[28] Kalvegren H, Skoglund C, Helldahl C, Lerm M, Grenegard M, Bengtsson T. Toll-like receptor 2 stimulation of platelets is mediated by purinergic P2X1-dependent Ca2+ mobilisation, cyclooxygenase and purinergic P2Y1 and P2Y12 receptor activation. Thromb Haemost 2010;103:398–407.
[29] Assinger A, Laky M, Schabbauer G, Hirschl AM, Buchberger E, Binder BR, et al. Efficient phagocytosis of periodontopathogens by neutrophils requires plasma factors, platelets and TLR2. J Thromb Haemost 2011;9:799–809.
[30] Rex S, Beaulieu LM, Perlman DH, Vitseva O, Blair PS, McComb ME, et al. Immune versus thrombotic stimulation of platelets differentially regulates signalling pathways, intracellular protein-protein interactions, and alpha-granule release. Thromb Haemost 2009;102:97–110.
[31] Zhang G, Han J, Welch EJ, Ye RD, Voyno-Yasenetskaya TA, Malik AB, et al. Lipopolysaccharide stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway. J Immunol 2009;182:7997–8004.
[32] Stahl AL, Svensson M, Morgelin M, Svanborg C, Tarr PI, Mooney JC, et al. Lipopolysaccharide from enterohemorrhagic Escherichia coli binds to platelets through TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. Blood 2006;108:167–76.
[33] Brown GT, McIntyre TM. Lipopolysaccharide signaling without a nucleus: kinase cascades stimulate platelet shedding of proinflammatory IL-1beta-rich microparticles. J Immunol 2011;186:5489–96.
[34] Montrucchio G, Bosco O, Del Sorbo L, Fascio Pecetto P, Lupia E, Goffi A, et al. Mechanisms of the priming effect of low doses of lipopoly-saccharides on leukocyte-dependent platelet aggregation in whole blood. Thromb Haemost 2003;90:872–81.
[35] Sheu JR, Hung WC, Kan YC, Lee YM, Yen MH. Mechanisms involved in the antiplatelet activity of Escherichia coli lipopolysaccharide in human platelets. Br J Haematol 1998;103:29–38.
[36] Etulain J, Negrotto S, Carestia A, Pozner RG, Romaniuk MA, D'Atri LP, et al. Acidosis downregulates platelet haemostatic functions and promotes neutrophil proinflammatory responses mediated by platelets. Thromb Haemost 2012;107:99–110.
[37] Pillitteri D, Bassus S, Boller K, Mahnel R, Scholz T, Westrup D, et al. Thrombin-induced interleukin 1beta synthesis in platelet suspensions: impact of contaminating leukocytes. Platelets 2007;18:119–27.
[38] Berthet J, Damien P, Hamzeh-Cognasse H, Arthaud CA, Eyraud MA, Zeni F, et al. Human platelets can discriminate between various bacterial LPS isoforms via TLR4 signaling and differential cytokine secretion. Clin Immunol 2012;145:189–200.
[39] Italiano Jr JE, Battinelli EM. Selective sorting of alpha-granule proteins. J Thromb Haemost 2009;7(Suppl. 1):173–6.
[40] Andonegui G, Kerfoot SM, McNagny K, Ebbert KV, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. Blood 2005;106:2417–23.
[41] Vieira-de-Abreu A, Campbell RA, Weyrich AS, Zimmerman GA. Platelets: versatile effector cells in hemostasis, inflammation, and the immune continuum. Semin Immunopathol 2012;34:5–30.
[42] Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler Thromb Vasc Biol 2012;32:1777–83.
[43] Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, et al. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem 1997;272:21096–103.
[44] Swinney DC, Xu YZ, Scarafia LE, Lee I, Mak AY, Gan QF, et al. A small molecule ubiquitination inhibitor blocks NF-kappa B-dependent cytokine expression in cells and rats. J Biol Chem 2002;277:23573–81.