

Published in final edited form as:

*J Immunol.* 2009 June 15; 182(12): 7997–8004. doi:10.4049/jimmunol.0802884.

## LPS stimulates platelet secretion and potentiates platelet aggregation via TLR4/MyD88 and the cGMP-dependent protein kinase pathway<sup>1</sup>

Guoying Zhang<sup>\*,†</sup>, Jingyan Han<sup>\*</sup>, Emily J. Welch<sup>\*</sup>, Richard D. Ye<sup>\*</sup>, Tatyana A. Voyno-Yasenetskaya<sup>\*,‡</sup>, Asrar B. Malik<sup>\*,‡</sup>, Xiaoping Du<sup>\*</sup>, and Zhenyu Li<sup>\*,†,2</sup>

<sup>\*</sup> Department of Pharmacology, College of Medicine, University of Illinois, Chicago, Illinois 60612

<sup>‡</sup> Center for Lung and Vascular Biology, College of Medicine, University of Illinois, Chicago, Illinois 60612

<sup>†</sup> Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky, Lexington, Kentucky 40536-0200

### Abstract

Bacterial lipopolysaccharide (LPS) induces rapid thrombocytopenia, hypotension and sepsis. Although growing evidence suggests that platelet activation plays a critical role in LPS-induced thrombocytopenia and tissue damage, the mechanism of LPS-mediated platelet activation is unclear. Here we show that LPS stimulates platelet secretion of dense and  $\alpha$  granules as indicated by ATP release and P-selectin expression, and thus enhances platelet activation induced by low concentrations of platelet agonists. Platelets express components of the LPS receptor-signaling complex, including Toll-like receptor (TLR4), CD14, MD2, and MyD88, and the effect of LPS on platelet activation was abolished by an anti-TLR4 blocking antibody or TLR4 knockout, suggesting that the effect of LPS on platelet aggregation requires the TLR4 pathway. Furthermore, LPS-potentiated thrombin- and collagen-induced platelet aggregation and FeCl<sub>3</sub>-induced thrombus formation were abolished in MyD88 knockout mice. LPS also induced cGMP elevation, and the stimulatory effect of LPS on platelet aggregation was abolished by inhibitors of nitric oxide synthase (NOS) and the cGMP-dependent protein kinase (PKG). LPS-induced cGMP elevation was inhibited by an anti-TLR4 antibody or by TLR4 deficiency, suggesting that activation of the cGMP/PKG pathway by LPS involves the TLR4 pathway. Taken together, our data indicate that LPS stimulates platelet secretion and potentiates platelet aggregation through a TLR4/MyD88 and cGMP/PKG-dependent pathway.

### Introduction

Bacteria-derived lipopolysaccharide (LPS) plays a fundamental role in sepsis. Following its release into the bloodstream, LPS forms a complex with LPS-binding protein (LBP) (1,2). This complex binds to CD14, a high-affinity LPS receptor present on the surface of several types

<sup>1</sup>This work is supported by AHA National Scientist Development Grant 0430095N and AHA Midwest affiliate Grand-in-Aid 0855698G (to Z.L.), and grants, HL68819, HL62350, and 080264, from National Institutes of Health/National Heart Lung and Blood Institute (to X.D.), and in part by the Centers of Biomedical Research Excellence (COBRE) in Obesity and Cardiovascular Disease P20 RR021954-01A1, from National Institutes of Health/the National Center for Research Resources (NCRR).

<sup>2</sup>Address correspondence and reprint requests to Dr. Zhenyu Li, Division of Cardiovascular Medicine, The Gill Heart Institute, 741 South Limestone Street, BBSRB Building, Rm B251, University of Kentucky, Lexington, Kentucky 40536-0200, zhenyuli08@uky.edu.

#### Disclosures

The authors have no financial conflict of interest.

of cells (3), and induces cellular responses through TLR4, the first-discovered mammalian homologue of *Drosophila* Toll (4,5). Recognition of LPS by TLR4 requires an extracellular adaptor protein, MD2. TLR4-induced intracellular signaling requires multiple adaptor proteins, including myeloid differentiation factor 88 (MyD88), the MyD88 adaptor-like protein (Mal), TIR-containing adaptor molecule (TRIF), and TRIF-related adapter molecule (TRAM) (6). Although TLR4 is the principal signal transducer for most types of LPS and TLR2 is a major receptor for lipoteichoic acid (LTA) from Gram-positive bacteria, TLR2 is also a signal transducer for at least some gram negative bacteria (7–10). TLR2 is expressed in platelets (11,12). Signal transduction by TLR2 also requires MyD88 pathway. Whether the entire LPS receptor-signaling complex is physically and functionally present in blood platelets remains unclear.

Patients with sepsis are often thrombocytopenic, and intravenous injection of LPS in mice also induces rapid thrombocytopenia (13,14). Under these conditions, platelet aggregates are found in lung and liver microvasculature (15,16). Recently, TLR4 has been found to be expressed in platelets and play important roles in LPS-induced thrombocytopenia (17,18). LPS enhances microvascular thrombosis in wild type mice, but not TLR4 deficient mice (19). Furthermore, infused platelets from wild type but not from TLR4 knockout mice accumulate in the lungs of LPS-treated wild type mice (17). Despite these *in vivo* data, several studies suggest that LPS does not affect human platelet function, while other studies report that LPS inhibits human platelets *in vitro* (19–22). Stahl et al recently reported that LPS activates the ligand binding function of integrin  $\alpha_{IIb}\beta_3$  (23). Thus, it remains controversial whether LPS directly induces platelet activation. Furthermore, it is unclear how TLR4 transmits LPS signals leading to platelet activation, and whether platelets express the necessary components of the TLR4 signaling complex. In this study, our experimental data suggest that LPS primarily stimulates platelet secretion of granule contents and thus enhances integrin-dependent platelet aggregation induced by multiple stimuli. We show that the components of the TLR4-MyD88 receptor-signaling complex required for LPS signaling are present in platelets. Importantly, we demonstrate that LPS-mediated platelet activation requires TLR4/MyD88-dependent activation of the nitric oxide (NO) and cGMP-dependent protein kinase pathway.

## Materials and Methods

### Reagents

LPS (*Escherichia coli* 0111:B4 and 055:B5) and the PKG inhibitor Rp-pCPT-cGMPS were purchased from Calbiochem. FeCl<sub>3</sub>, LPS (*Escherichia coli* 0127:B8), the purified LPS (*Escherichia coli* 0111:B4, Cat# L4391), a monoclonal antibody against  $\beta$ -actin (AC74) and N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) were from Sigma. Kdo(2)-Lipid A was a generous gift from Dr. Andrew J. Morris, University of Kentucky. Polyclonal antibodies against human TLR4 (H-80) or MyD88 (HFL-296), and monoclonal antibodies against CD14 (UCH-M1) or a complex of TLR4-MD2 (HTA125) were purchased from Santa Cruz. Blocking monoclonal antibodies against human TLR4 (HTA125) or TLR2 (Clone T2.5) were from eBioscience.  $\alpha$ -thrombin was from Enzyme Research Laboratories. Collagen and luciferin-luciferase reagent were purchased from Chronolog. MyD88 knockout mice were obtained from Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) (24,25). MyD88 knockout and wild type mice obtained from heterozygous breeding were used for the experiments. TLR4 deficient mouse strain C57BL/10ScCr and wild type control C57BL/10J were from Jackson laboratory. Mice were bred and maintained in the University of Illinois Animal Care Facility following institutional and National Institutes of Health guidelines after approval by the Animal Care Committee.

### Preparation of washed platelets

Fresh blood from healthy volunteers was anticoagulated with 1/7<sup>th</sup> volume of acid-citrate dextrose (ACD) (85 mM trisodium citrate, 110 mM dextrose, and 78 mM citric acid) as described previously (27). For the preparation of mouse platelets, 6–8 week old mice of either sex were anesthetized with an intraperitoneal injection of pentobarbital and blood was drawn from the inferior vena cava. Blood from 5 to 6 mice of either genotype was pooled using 1/7 volume of ACD (85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid) as anticoagulant and platelets were isolated by differential centrifugation as previously described (26). Platelets were washed twice with CGS buffer (sodium chloride 0.12 M, trisodium citrate 0.0129 M, and D-glucose 0.03 M, pH 6.5), resuspended in freshly made but not sterile Tyrode's buffer and allowed to rest for at least 1 hour at 37°C before use (28). In platelet aggregation experiments using platelet-rich plasma (PRP), 1/10 volume of 3.8% trisodium citrate was used as anticoagulant.

### Platelet aggregation and secretion

Platelet aggregation was measured in a turbidometric platelet aggregometer (Chronolog) at 37°C with stirring (1000 rpm) (26). To examine the effect of LPS on platelet activation, various concentrations of LPS were added simultaneously with or without low concentrations of platelet agonists to induce platelet aggregation. ATP release was monitored in parallel with platelet aggregation. To examine the effect of LPS on ATP release, washed human platelets were incubated with LPS at room temperature for 10 min, ATP in the supernatant was measured by addition of luciferin-luciferase reagent. Platelets were also incubated with 0.025 U/ml thrombin at room temperature for 10 min as a positive control. Quantification was performed using the ATP standard. Statistical analysis was performed using t-test.

### P-selectin Expression

Washed human platelets were incubated with LPS or thrombin in the presence of a monoclonal anti-human P-selectin antibody, SZ51 (29), or control mouse IgG, for 30 min at 22 °C. After washed once with PBS, the platelets were incubated with a FITC-conjugated rabbit anti-mouse IgG antibody. P-selectin expression was analyzed using a FACScalibur flow cytometer.

### Detection of TLR4 and MyD88 expression in platelets by western blot

Washed human platelets or leukocytes were solubilized in SDS-PAGE sample buffer. Western blot was performed as described previously (27) with polyclonal anti-TLR4 or MyD88 antibodies.

### Detection of TLR4, MD2 and CD14 on the platelet surface by flow cytometry

Human platelets were incubated with a polyclonal antibody against human TLR4 (H-80), or a monoclonal antibody (HTA125) that recognize TLR4/MD2 complex, or a monoclonal antibody against human CD14 (UCH-M1), for 30 min at 22 °C. Platelets were also incubated with control IgG for 30 min. After washed once with PBS, platelets were incubated with a FITC-conjugated anti-rabbit or mouse IgG antibody. TLR4, TLR4/MD2, or CD14 expression was analyzed using a FACScalibur flow cytometer.

### Measurement of platelet cGMP levels

Washed human platelets, resuspended in modified Tyrode's buffer, were incubated with various concentrations of LPS for 5 minutes in platelet aggregometer with stirring (1000 rpm) at 37°C. Washed platelets were incubated with thrombin (0.02 U/ml), LPS, or thrombin plus LPS at 37°C in a platelet aggregometer for 5 minutes. Washed human platelets were also incubated with various concentrations of LPS for two minutes at 22°C. The reaction was

stopped by addition of ice cold 12% (w/v) trichloroacetic acid. The samples were then centrifuged at 2000g for 15 minutes at 4°C, and the supernatant extracted 4 times with 5 volumes of water saturated diethyl ether. The samples were lyophilized and cGMP concentrations were determined using a cGMP enzyme immunoassay kit from Amersham-Pharmacia Biotech (27). Results are expressed as mean  $\pm$  standard deviation. Statistical significance between groups was determined by t-test.

### In Vivo Thrombosis

FeCl<sub>3</sub>-injured carotid artery thrombus formation was performed as described previously (30). Briefly, 0.5  $\mu$ l of 4% FeCl<sub>3</sub> was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the carotid artery for 3 min to induce vessel damage. LPS (0.5 mg/kg weight in 0.1 ml saline) or saline, was injected into the fundus oculi of the mice 1 min after the initiation of carotid artery injury. Time to occlusion was calculated as a difference in time between the removal of the filter paper and stable occlusion (no blood flow for 2 min). Statistical analysis was performed using the Mann-Whitney test for the evaluation of differences in median occlusion time.

## Results

### LPS enhances platelet aggregation induced by low concentrations of platelet agonists

To determine whether LPS directly induces platelet activation, we examined the effect of LPS on platelet aggregation using washed human platelets. When used alone, LPS (0111:B4, from Calbiochem, contaminated  $\leq$ 2% proteins) up to 100  $\mu$ g/ml failed to induce aggregation (Fig. 1A). However, when added simultaneously with subthreshold concentrations of the platelet agonists thrombin or collagen, LPS significantly enhanced platelet aggregation (Fig. 1B, 1C, and 1D). These results were confirmed with purified LPS (0111:B4, Sigma Cat#: L4391, contaminated proteins <1%) (data not shown). Recently, a well defined and highly pure LPS, Kdo(2)-Lipid A, has been shown to activate the TLR4 pathway with a similar bioactivity as LPS (31,32). To further verify the stimulatory effect of LPS on platelet activation and exclude the possibility that the stimulatory effect of LPS on platelet activation is caused by contaminated proteins, we examined the effect of Kdo(2)-Lipid A on platelet activation. Similar as LPS, Kdo(2)-Lipid A enhanced platelet aggregation induced by low dose thrombin (Fig. 1E). Thus, LPS can directly interact with platelets and plays a stimulatory role that synergizes with low concentrations of platelet agonists to induce platelet aggregation.

To determine whether LPS is also able to promote platelet aggregation in the presence of plasma, we examined the effect of LPS on platelet aggregation in human platelet-rich plasma (PRP). We found that LPS also significantly enhanced collagen-induced platelet aggregation in PRP (Fig. 1F). The concentration of LPS required for potentiating platelet aggregation in plasma (1  $\mu$ g/ml) is significantly lower than that required for washed platelets (5–10  $\mu$ g/ml). We also compared LPS from different sources, and found that their potency in potentiating collagen-induced platelet aggregation varies with the order of 0127:B8 > 0111:B4 > 055:B5 (Fig. 1F).

### The effects of LPS on platelet granule secretion

Platelet secretion plays a critical role in potentiating platelet activation induced by low dose agonists. To determine whether platelet secretion accounted for the potentiating effect of LPS on platelet aggregation, we examined LPS-induced ATP release in the human platelets, which indicates the secretion of dense granules. LPS alone is sufficient to induce the release of ATP (ATP concentrations were  $573.33 \pm 62.22$  (basal level) versus  $986.67 \pm 97.78$  for 0111:B4 and  $1186.67 \pm 62.22$  for 0127:B8 nmol/L ATP in the supernatant of solvent-treated platelets versus LPS-stimulated platelets) (Fig. 2A), although the amount of ATP release induced by LPS

stimulation is much lower than that induced by platelet agonists, such as thrombin (thrombin, at 0.025 U/ml, induced about 38 fold increase of ATP release than the basal level) (supplemental Fig. 1). The low level secretion induced by LPS stimulation may explain why LPS alone is insufficient to induce platelet aggregation. Similarly, LPS alone induced P-selectin expression in human platelets, indicating that LPS also stimulates  $\alpha$ -granule secretion (Fig. 2B and 2C). Thus, LPS stimulates platelet secretion of both dense and  $\alpha$ -granules.

### **Platelets express components of the LPS receptor-signal complex, TLR4, MD-2, CD14, and MyD88**

Platelets have been previously reported to express TLR4 (12,17,18). Consistently, we detected TLR4 in human platelets by western blot analysis with a rabbit anti-TLR4 polyclonal antibody (Fig. 3A). The expression of TLR4 on human platelets was further confirmed by flow cytometry using the anti-TLR4 polyclonal antibody (Fig. 3B). In addition, we found that other members of the TLR4 receptor complex, MD-2 (Fig. 3C) and CD14 (Fig. 3D), are also present on the surface of platelet as analyzed by flow cytometry. Furthermore, the intracellular adaptor protein MyD88 was detected in human platelet lysates. It is important to note that the MyD88 detected in our platelet preparation is unlikely to be from contaminated WBCs, since the MyD88 expression level in the platelet preparation was much higher than that detected in 10 times as much the leukocytes as the contamination level (0.016% as estimated by HEMAVET HV950FS multispecies hematology analyzer) (Fig. 3E). Thus, platelets express important components of the TLR4 signaling complex, including TLR4, CD14, MD-2, and the adaptor protein MyD88.

### **LPS-mediated platelet activation requires TLR4/MyD88**

To assess the role of TLR4 in LPS-induced platelet responses, human platelets were pre-incubated with an anti-human TLR4 blocking antibody or control IgG, and then exposed to a subthreshold concentration of thrombin, in the presence of LPS, to induce platelet aggregation and secretion. LPS significantly enhanced low dose thrombin-induced platelet aggregation and ATP release in control IgG-treated platelets, but not in anti-TLR4 antibody-treated platelets (Fig. 4A). The stimulatory effect of LPS on platelet aggregation and secretion was also abolished in TLR4-deficient platelets (Fig. 4B and 4C). These results suggest that the stimulatory effect of LPS on platelet activation is mainly TLR4 dependent. Also, LPS enhanced aggregation and secretion of wild type mouse platelets, but not MyD88 deficient platelets (Fig. 5A and 5B). MyD88 plays important roles not only in TLR receptor signaling, but also in IL-1 signaling (33). It has been recently reported that LPS stimulation induces IL-1 synthesis in platelets (34). Thus, to exclude the possibility that the effect of MyD88 deficiency on LPS-promoted platelet activation results from its role in IL-1 signaling, we examine whether IL-1 is involved in LPS-potentiated platelet aggregation. LPS-potentiated platelet aggregation was not affected by a recombinant human interleukin-1 receptor antagonist, IL-1ra (from Imgenex) (supplemental Fig. 2), suggesting that IL-1 is not required for LPS-promoted platelet activation. Thus, these results suggest that the stimulatory role of LPS in platelet aggregation requires TLR4/MyD88 signaling.

### **LPS promotes thrombus formation in vivo**

To investigate the role of LPS in platelet-dependent thrombosis *in vivo*, we determined the effect of LPS on FeCl<sub>3</sub>-injured carotid artery thrombus formation. Fig. 5C shows that the median time from injury to formation of stable occlusive thrombus in LPS-treated mice (184.0 seconds) is significantly shortened compared to that of control mice (775.0 Seconds) ( $p < 0.001$ ). Thus, LPS significantly aggravates thrombus formation *in vivo*. In contrast, LPS failed to accelerate the FeCl<sub>3</sub>-induced *in vivo* thrombosis in MyD88 knockout mice (Fig 5C). Therefore,

the TLR-associated adapter protein MyD88 is critical to the role of LPS in accelerating FeCl<sub>3</sub>-induced arterial thrombosis.

### LPS induces cGMP elevation in human platelets

The characteristics of LPS-mediated potentiation of platelet activation is similar to the recently identified stimulatory roles of NO and cGMP in platelet activation (29,30,35), in that both LPS and cGMP primarily stimulate secretion of platelet granules but not platelet aggregation. LPS stimulation of nitric oxide (NO) production has been shown in other cell types, resulting in the activation of soluble guanylyl cyclase (sGC) and produces cGMP. To investigate whether the stimulatory effect of LPS on platelet activation involves LPS-mediated activation of the cGMP pathway, washed human platelets were stimulated with LPS, and intracellular cGMP levels were measured by a solid phase enzyme-linked immunoassay. LPS dose-dependently enhanced cGMP levels in human platelets (Fig. 6A and 6B). LPS-induced cGMP elevation was partially but significantly reduced in TLR4-deficient platelets (Fig. 6C), suggesting the existence of both TLR4-dependent and TLR4-independent pathways mediating LPS-induced cGMP elevation.

TLR2 has been shown to be expressed in platelets. To determine whether TLR2 is responsible for LPS-induced, TLR4-independent, cGMP elevation and platelet activation, we examined the effect of an anti-TLR2 blocking antibody on LPS-induced cGMP production and platelet aggregation. Fig. 6D shows that LPS-induced cGMP elevation was inhibited by both anti-TLR4 and anti-TLR2 antibodies. Furthermore, the effect of LPS on platelet aggregation was also blocked by the anti-TLR2 antibody (Fig 6E). Thus, these data indicate that TLR2 is also involved in LPS signaling in platelets.

### PKG plays an important role in LPS-induced platelet activation

To determine whether NO/cGMP/PKG pathway is important in LPS stimulation of platelet activation, we examined the effect of the inhibitors of NOS and PKG on LPS-mediated potentiation of platelet aggregation induced by low doses of thrombin or collagen. The enhancing effect of LPS on platelet aggregation induced by low concentrations of thrombin (Fig. 6F) was abolished by the NOS inhibitor, L-NAME, or the PKG inhibitor, Rp-pCPT-cGMPS, indicating that the PKG pathway is important in LPS-mediated potentiation of platelet activation.

## Discussion

In this study, we show that platelets express the necessary components of LPS receptor-signaling complex, including CD14, TLR4/MD2, and MyD88. We have provided direct evidence that LPS stimulates platelet secretion and amplifies platelet aggregation mainly via the TLR4/MyD88 dependent mechanisms. Our data also reveal a novel signaling pathway mediating LPS-induced platelet activation, in which LPS binding to its platelet receptor induces cGMP production and activates PKG, leading to platelet secretion and thus amplification of aggregation.

We have discovered here that LPS promotes platelet activation by inducing secretion of contents of both  $\alpha$  and dense granules, and thus amplifying secretion-dependent platelet aggregation. Previously, it was known that platelets are involved in the pathogenesis of severe sepsis (36–38), and that LPS stimulates thrombosis and formation of platelet microaggregates *in vivo*. It is also known that LPS stimulates the interaction between platelets and neutrophils, leading to robust neutrophil activation via TLR4-dependent mechanisms (18,39,40). However, it is unclear whether the *in vivo* effect of LPS on platelet activation is due to the direct response of platelets to LPS, and the mechanisms of platelet activation and activation of leukocyte-

platelet interactions in patients with sepsis is poorly understood. P-selectin has been shown to play an important role in LPS binding to platelets (23) and LPS-mediated platelet-leukocyte interaction (41). However, it is controversial whether LPS is able to induce P-selectin expression in platelets. Although P-selectin was not detected by LPS stimulation in some previous reports (12,17,19,39), recent studies suggest that LPS is able to induce platelet secretion of P-selectin (34,42). It is not clear what caused this discrepancy. In this study, we confirmed that LPS is capable of inducing P-selectin expression on platelet surface. Although the potency to induce P-selectin expression varies between different sources of LPS, all the tested LPS preparations from different sources induced P-selectin expression in human platelets in our hands (data not shown). We found that the resting state of platelets after preparation is an important factor affecting the detection of LPS-induced P-selectin expression, as partially activated platelets already express substantial levels of P-selectin. Under these conditions, the LPS-induced increase in P-selectin expression is no longer detectable. Our results suggest that LPS not only induces platelet secretion from  $\alpha$  granules, but also induces platelet secretion from dense granules. ADP, secreted from dense granules, plays an important role in inducing integrin activation and platelet aggregation mainly through its P2Y1 and P2Y12 receptors. Thus, our findings that LPS stimulates platelet secretion and synergizes with platelet agonists in platelet activation may be a mechanism that explains the role of platelets in exacerbating LPS-induced sepsis. A recent report indicates that coagulation initiated by cytokines plays an important role in amplifying inflammatory response in sepsis (43). It has also been reported that stimulation of platelets with LPS induces release of cytokines such as interleukin 8 (IL-8), EGF, and TGF beta (42). Thus, platelet activation induced by LPS during sepsis may not only contribute to coagulation, but also contribute to inflammation during sepsis.

We conclude that LPS stimulates platelet secretion and aggregation via the TLR4/MyD88 receptor-signaling complex. This conclusion is supported by our data that TLR4, MD2, CD14 and MyD88 are all present in platelets, and that the stimulatory effect of LPS is abolished by an anti-TLR4 antibody and in TLR4- or MyD88-deficient platelets. Our results are consistent with the findings that TLR4 is expressed on platelets (12,17,18), but further shows that other necessary components of the TLR4 receptor-signaling complex are also present in platelets. We found that the high affinity LPS receptor CD14 is present on platelet surface, which contradicts with previous reports that CD14 is not expressed in platelets (34). While the reason for this discrepancy is not clear, we cannot exclude the possibility that the detected CD14 may be from plasma CD14 since plasma contains microgram levels of soluble CD14. Our results agree with previous findings that platelet TLR4 plays an important role in LPS-induced thrombocytopenia and thrombus formation *in vivo* (17). Furthermore, the concentration of LPS required for promoting platelet aggregation in the presence of plasma is only 1  $\mu\text{g/ml}$  in our studies, which is known to be achievable in patients with severe sepsis (1 to 10  $\mu\text{g/ml}$ ) (44), in whom DIC and severe thrombocytopenia are more often seen (LPS is released from the local infected area, thus the local LPS concentration could be even higher than detected in systemic concentrations). Our results, together with previous findings, indicate that TLR4/MyD88-signaling complex in platelets is important in the development of thrombotic complications in sepsis and in the pathogenesis of sepsis. We found that the concentration of LPS required for potentiating platelet aggregation in plasma (1  $\mu\text{g/ml}$ ) is significantly lower than that required for washed platelets (5–10  $\mu\text{g/ml}$ ) (Fig. 1 and 2A), suggesting the presence of a plasma co-factor sensitizing platelet to low concentrations of LPS.

We conclude that LPS stimulates platelets via the cGMP-dependent signaling pathway. This is supported by the data that LPS induces cGMP elevation in platelets, and that the stimulatory effect of LPS on platelets is inhibited by either the NOS inhibitor L-NAME or the PKG inhibitor Rp-pCPT-cGMPs. We have recently reported that the NO-cGMP-PKG pathway plays biphasic roles in platelet activation. At high concentrations, NO and cGMP inhibit platelets via a

mechanism that involves cGMP-dependent protein kinase I (45,46) and cAMP (47). However, during platelet activation, endogenously produced NO at low concentrations and cGMP can promote platelet activation (27,30,48). Importantly, the NO-cGMP-PKG pathway is similar to the TLR4/MyD88 complex in that both the NO-cGMP pathway and LPS stimulate secretion of platelet granules and potentiate platelet aggregation in the presence of low concentrations of agonists, but alone are not sufficient to cause platelet aggregation (29). Therefore, we have discovered a novel pathway of LPS-mediated platelet activation. In this pathway, LPS, by interacting with TLR4/MyD88 receptor-signaling complex, activates the NO-cGMP pathway, stimulating platelet granule secretion, leading to potentiation and amplification of platelet activation and aggregation. Although the potency to induce cGMP elevation is different between different sources of LPS, all tested LPS preparations increased cGMP concentration in platelets. Consistently, 0127:B8 is the most potent in promotion platelet aggregation and induced maximal cGMP production. In contrast, 055:B5 was the least potent in promotion platelet aggregation and induced the least amount of cGMP. Our data that LPS-induced cGMP elevation is only partially inhibited in TLR4 deficient platelets or anti-TLR4 antibody treated human platelets suggest that there is a TLR4-independent signaling pathway mediating LPS-induced activation of cGMP/PKG pathway. Platelets express functional TLR2 (11,12). In addition to TLR4, TLR2 has been shown to be a signal transducer for some sources of LPS (7–10). We found that LPS-induced cGMP elevation is significantly inhibited by an anti-TLR2 blocking antibody, and the potentiation of platelet aggregation by LPS is inhibited by anti-TLR2 antibody (Fig. 6). These results indicate that TLR2 is also involved in LPS-mediated cGMP elevation and platelet activation. TLR2 signaling also requires MyD88. Therefore, it is not surprising that LPS-promoted platelet activation and thrombosis is abolished in MyD88 deficient mice. It is interesting to note that our finding that LPS stimulates platelet activation via the cGMP/PKG pathway may provide an explanation of previous controversy whether LPS stimulates or inhibits platelet activation. Previous studies showing the inhibitory effect of LPS on platelets allowed prolonged preincubation of high dose of LPS with platelets (19,22). Since LPS induces cGMP elevation, it is possible that prolonged preincubation of high dose of LPS inhibited platelet activation in a way similar to the inhibitory effect of preincubation of high concentrations of cGMP. We show that LPS promotes platelet aggregation when added immediately before or after the agonist stimulation, which is also similar to the way endogenous cGMP stimulates platelet activation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Dr. Feng Qian for excellent technical assistance.

## References

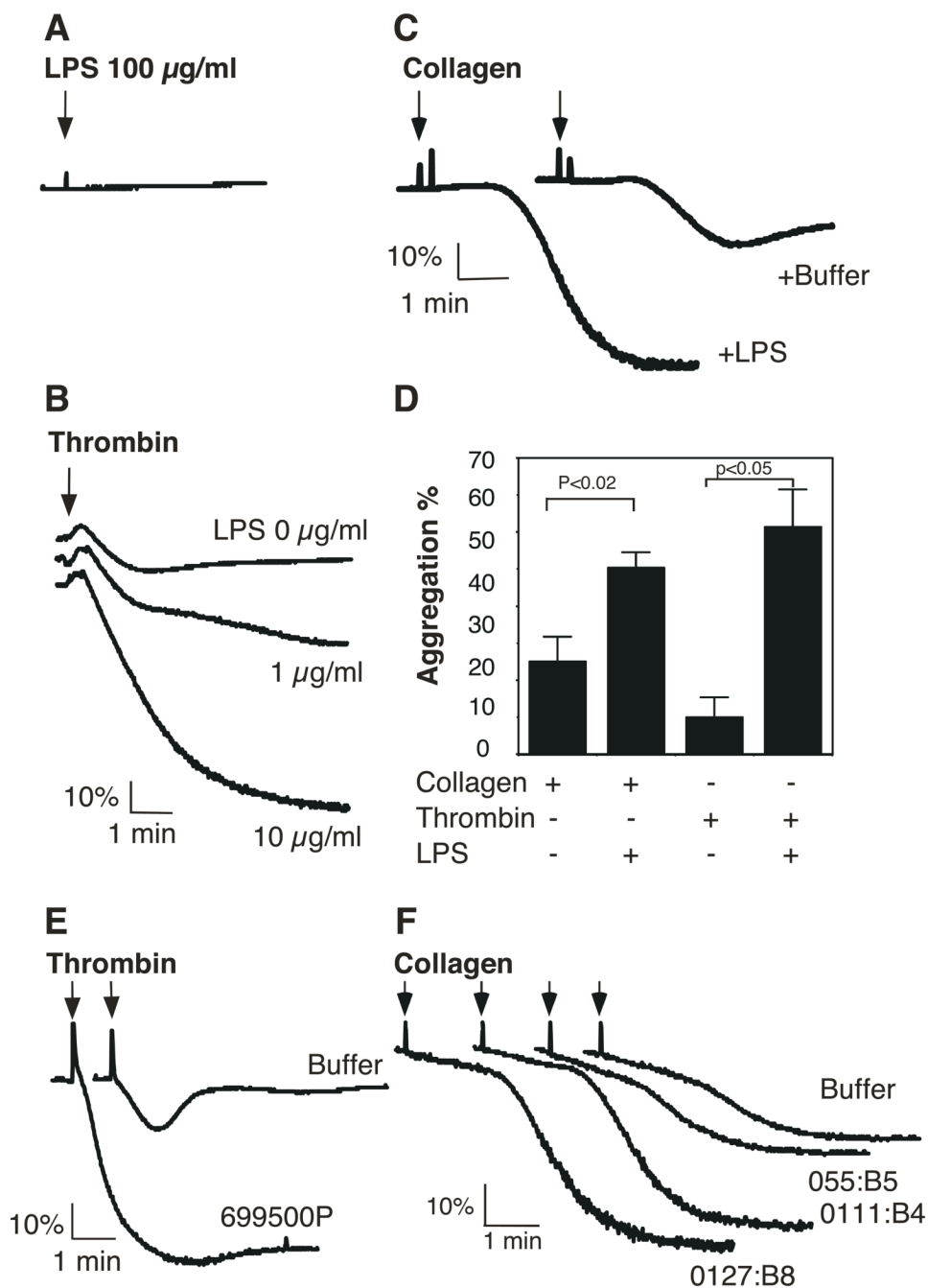
1. Tobias PS, Mathison JC, Ulevitch RJ. A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. *J Biol Chem* 1988;263:13479–13481. [PubMed: 3138236]
2. Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. *Science* 1990;249:1429–1431. [PubMed: 2402637]
3. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990;249:1431–1433. [PubMed: 1698311]
4. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394–397. [PubMed: 9237759]



5. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;282:2085–2088. [PubMed: 9851930]
6. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 2004;113:153–162. [PubMed: 15379975]
7. Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, Ulevitch RJ. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat Immunol* 2001;2:346–352. [PubMed: 11276206]
8. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, Howald WN, Way SS, Hajjar AM. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect Immun* 2004;72:5041–5051. [PubMed: 15321997]
9. Erridge C, Pridmore A, Eley A, Stewart J, Poxton IR. Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. *J Med Microbiol* 2004;53:735–740. [PubMed: 15272059]
10. Nemoto E, Darveau RP, Foster BL, Nogueira-Filho GR, Somerman MJ. Regulation of cementoblast function by *P. gingivalis* lipopolysaccharide via TLR2. *J Dent Res* 2006;85:733–738. [PubMed: 16861291]
11. 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;83:196–198. [PubMed: 15748217]
12. Ward JR, Bingle L, Judge HM, Brown SB, Storey RF, Whyte MK, Dower SK, Buttle DJ, Sabroe I. 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;94:831–838. [PubMed: 16270639]
13. Davis RB, Meeker WR, Mc QD. Immediate effects of intravenous endotoxin on serotonin concentrations and blood platelets. *Circ Res* 1960;8:234–239. [PubMed: 13814510]
14. Vincent JL, Yagushi A, Pradier O. Platelet function in sepsis. *Crit Care Med* 2002;30:S313–317. [PubMed: 12004253]
15. Endo Y, Shibazaki M, Nakamura M, Takada H. Contrasting effects of lipopolysaccharides (endotoxins) from oral black-pigmented bacteria and Enterobacteriaceae on platelets, a major source of serotonin, and on histamine-forming enzyme in mice. *J Infect Dis* 1997;175:1404–1412. [PubMed: 9180180]
16. Shibazaki M, Kawabata Y, Yokochi T, Nishida A, Takada H, Endo Y. Complement-dependent accumulation and degradation of platelets in the lung and liver induced by injection of lipopolysaccharides. *Infect Immun* 1999;67:5186–5191. [PubMed: 10496894]
17. Andonegui G, Kerfoot SM, McNagny K, Ebbert KV, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. *Blood* 2005;106:2417–2423. [PubMed: 15961512]
18. Aslam R, Speck ER, Kim M, Crow AR, Bang KW, Nestel FP, Ni H, Lazarus AH, Freedman J, Semple JW. Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production in vivo. *Blood* 2006;107:637–641. [PubMed: 16179373]
19. Rumbaut RE, Bellera RV, Randhawa JK, Shrimpton CN, Dasgupta SK, Dong JF, Burns AR. Endotoxin enhances microvascular thrombosis in mouse cremaster venules via a TLR4-dependent, neutrophil-independent mechanism. *Am J Physiol Heart Circ Physiol* 2006;290:H1671–1679. [PubMed: 16284241]
20. Nagayama M, Zucker MB, Beller FK. Effects of a variety of endotoxins on human and rabbit platelet function. *Thromb Diath Haemorrh* 1971;26:467–473. [PubMed: 4947419]
21. Abdelnoor AM, Kassem H, Bikhazi AB, Nowotny A. Effect of gram-negative bacterial lipopolysaccharide-derived polysaccharides, glycolipids, and lipopolysaccharides on rabbit and human platelets in vitro. *Immunobiology* 1980;157:145–153. [PubMed: 6997189]
22. Sheu JR, Hung WC, Su CH, Lin CH, Lee LW, Lee YM, Yen MH. The antiplatelet activity of *Escherichia coli* lipopolysaccharide is mediated through a nitric oxide/cyclic GMP pathway. *Eur J Haematol* 1999;62:317–326. [PubMed: 10359060]
23. Stahl AL, Svensson M, Morgelin M, Svanborg C, Tarr PI, Mooney JC, Watkins SL, Johnson R, Karpman D. Lipopolysaccharide from enterohemorrhagic *Escherichia coli* binds to platelets through

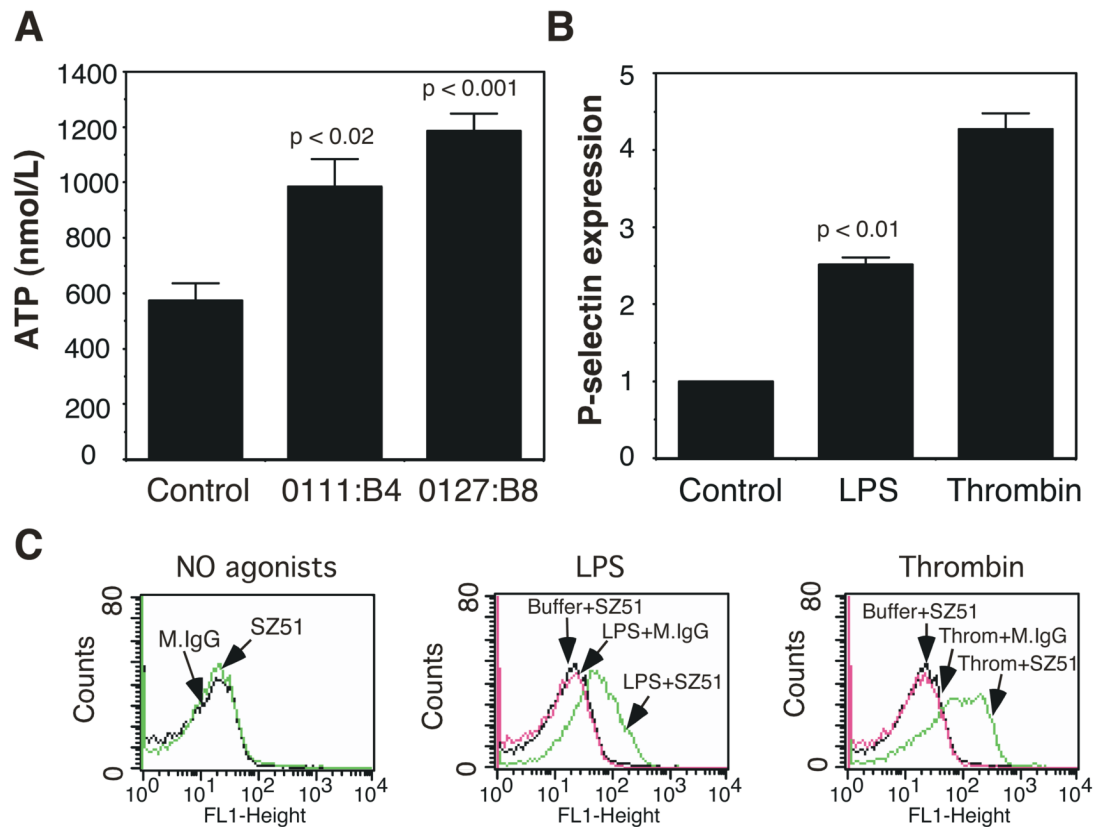
- TLR4 and CD62 and is detected on circulating platelets in patients with hemolytic uremic syndrome. *Blood* 2006;108:167–176. [PubMed: 16514062]
24. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999;11:115–122. [PubMed: 10435584]
  25. Fan J, Frey RS, Malik AB. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J Clin Invest* 2003;112:1234–1243. [PubMed: 14561708]
  26. Li Z, Zhang G, Le Breton GC, Gao X, Malik AB, Du X. Two waves of platelet secretion induced by thromboxane A2 receptor and a critical role for phosphoinositide 3-kinases. *J Biol Chem* 2003;278:30725–30731.
  27. Li Z, Xi X, Gu M, Feil R, Ye RD, Eigenthaler M, Hofmann F, Du X. A stimulatory role for cGMP-dependent protein kinase in platelet activation. *Cell* 2003;112:77–86. [PubMed: 12526795]
  28. Du XP, Plow EF, Frelinger AL 3rd, O’Toole TE, Loftus JC, Ginsberg MH. Ligands “activate” integrin alpha IIb beta 3 (platelet GPIIb-IIIa). *Cell* 1991;65:409–416. [PubMed: 2018974]
  29. Li Z, Zhang G, Marjanovic JA, Ruan C, Du X. A platelet secretion pathway mediated by cGMP-dependent protein kinase. *J Biol Chem* 2004;279:42469–42475. [PubMed: 15280395]
  30. Marjanovic JA, Li Z, Stojanovic A, Du X. Stimulatory roles of nitric-oxide synthase 3 and guanylyl cyclase in platelet activation. *J Biol Chem* 2005;280:37430–37438. [PubMed: 16144836]
  31. Raetz CR, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC Jr, Ribeiro AA, Murphy RC, Ulevitch RJ, Fearn C, Reichart D, Glass CK, Benner C, Subramaniam S, Harkewicz R, Bowers-Gentry RC, Buczynski MW, Cooper JA, Deems RA, Dennis EA. Kdo2-Lipid A of *Escherichia coli*, a defined endotoxin that activates macrophages via TLR-4. *J Lipid Res* 2006;47:1097–1111. [PubMed: 16479018]
  32. Sasaki H, White SH. Aggregation behavior of an ultra-pure lipopolysaccharide that stimulates TLR-4 receptors. *Biophys J* 2008;95:986–993. [PubMed: 18375521]
  33. Yamamoto M, Takeda K, Akira S. TIR domain-containing adaptors define the specificity of TLR signaling. *Mol Immunol* 2004;40:861–868. [PubMed: 14698224]
  34. Shashkin PN, Brown GT, Ghosh A, Marathe GK, McIntyre TM. Lipopolysaccharide is a direct agonist for platelet RNA splicing. *J Immunol* 2008;181:3495–3502. [PubMed: 18714022]
  35. Stojanovic A, Marjanovic JA, Brovkovich VM, Peng X, Hay N, Skidgel RA, Du X. A phosphoinositide 3-kinase-AKT-nitric oxide-cGMP signaling pathway in stimulating platelet secretion and aggregation. *J Biol Chem* 2006;281:16333–16339. [PubMed: 16613861]
  36. Zhao L, Ohtaki Y, Yamaguchi K, Matsushita M, Fujita T, Yokochi T, Takada H, Endo Y. LPS-induced platelet response and rapid shock in mice: contribution of O-antigen region of LPS and involvement of the lectin pathway of the complement system. *Blood* 2002;100:3233–3239. [PubMed: 12384422]
  37. Taylor FB, Collier BS, Chang AC, Peer G, Jordan R, Engellener W, Esmon CT. 7E3 F(ab’)2, a monoclonal antibody to the platelet GPIIb/IIIa receptor, protects against microangiopathic hemolytic anemia and microvascular thrombotic renal failure in baboons treated with C4b binding protein and a sublethal infusion of *Escherichia coli*. *Blood* 1997;89:4078–4084. [PubMed: 9166848]
  38. Pu Q, Wiel E, Corseaux D, Bordet R, Azrin MA, Ezekowitz MD, Lund N, Jude B, Vallet B. Beneficial effect of glycoprotein IIb/IIIa inhibitor (AZ-1) on endothelium in *Escherichia coli* endotoxin-induced shock. *Crit Care Med* 2001;29:1181–1188. [PubMed: 11395599]
  39. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD, Keys EM, Allen-Vercoe E, Deviney R, Doig CJ, Green FH, Kubers P. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* 2007;13:463–469. [PubMed: 17384648]
  40. Semple JW, Aslam R, Kim M, Speck ER, Freedman J. Platelet-bound lipopolysaccharide enhances Fc receptor-mediated phagocytosis of IgG-opsonized platelets. *Blood* 2007;109:4803–4805. [PubMed: 17299089]
  41. Montrucchio G, Bosco O, Del Sorbo L, Fascio Pecetto P, Lupia E, Goffi A, Omede P, Emanuelli G, Camussi G. Mechanisms of the priming effect of low doses of lipopoly-saccharides on leukocyte-dependent platelet aggregation in whole blood. *Thromb Haemost* 2003;90:872–881. [PubMed: 14597983]

42. Cognasse F, Hamzeh-Cognasse H, Lafarge S, Delezay O, Pozzetto B, McNicol A, Garraud O. Toll-like receptor 4 ligand can differentially modulate the release of cytokines by human platelets. *Br J Haematol* 2008;141:84–91. [PubMed: 18279456]
43. Niessen F, Schaffner F, Furlan-Freguia C, Pawlinski R, Bhattacharjee G, Chun J, Derian CK, Andrade-Gordon P, Rosen H, Ruf W. Dendritic cell PAR1-S1P3 signalling couples coagulation and inflammation. *Nature* 2008;452:654–658. [PubMed: 18305483]
44. Behre G, Schedel I, Nentwig B, Wormann B, Essink M, Hiddemann W. Endotoxin concentration in neutropenic patients with suspected gram-negative sepsis: correlation with clinical outcome and determination of anti-endotoxin core antibodies during therapy with polyclonal immunoglobulin M-enriched immunoglobulins. *Antimicrob Agents Chemother* 1992;36:2139–2146. [PubMed: 1444293]
45. Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Fassler R, Ruth P, Krombach F, Hofmann F. Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. *J Exp Med* 1999;189:1255–1264. [PubMed: 10209042]
46. Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits platelet recruitment. *J Clin Invest* 1997;100:350–356. [PubMed: 9218511]
47. Li Z, Ajdic J, Eigenthaler M, Du X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* 2003;101:4423–4429. [PubMed: 12576312]
48. Iafrati MD, Vitseva O, Tanriverdi K, Blair P, Rex S, Chakrabarti S, Varghese S, Freedman JE. Compensatory mechanisms influence hemostasis in setting of eNOS deficiency. *Am J Physiol Heart Circ Physiol* 2005;288:H1627–1632. [PubMed: 15563534]



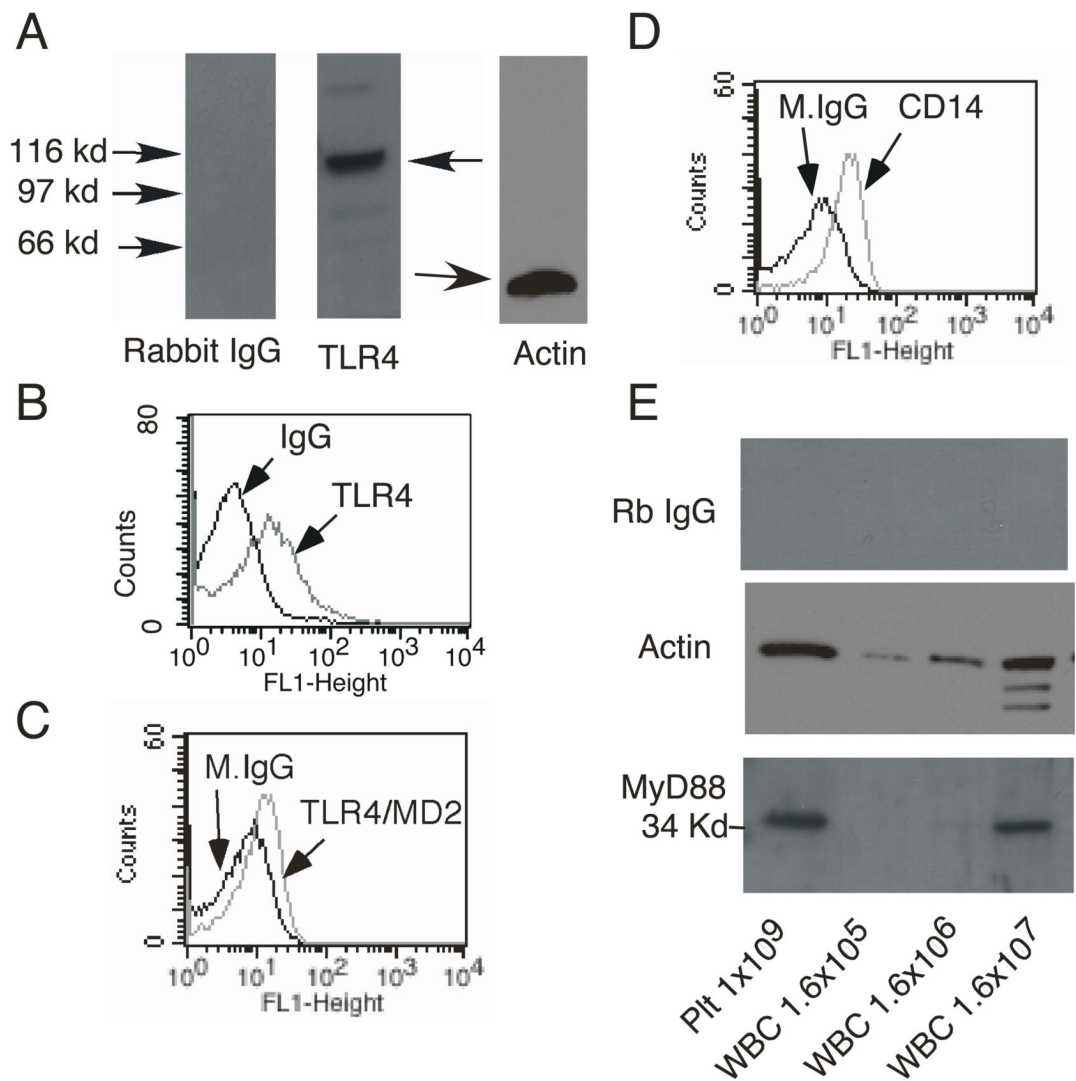
**Figure 1.** LPS potentiates platelet aggregation. *A*, LPS (0111:B4, 100  $\mu\text{g/ml}$ ) was added to washed human platelets and incubated in the aggregometer. *B*, A subthreshold concentration of thrombin (0.0185 U/ml) was added to washed human platelets immediately followed by addition of various concentrations of LPS (0111:B4) or buffer. *C*, A subthreshold concentration of collagen (0.3  $\mu\text{g/ml}$ ) was added to the washed human platelets followed by addition of LPS (0111:B4, 5  $\mu\text{g/ml}$ ) or buffer. *D*, Quantitative data (mean  $\pm$  SD) from four experiments described in *B* (LPS 10  $\mu\text{g/ml}$ ) and *C*. *E*, A subthreshold concentration of thrombin (0.0185 U/ml) was added to washed human platelets immediately followed by addition of Kdo(2)-Lipid A (10  $\mu\text{g/ml}$ ) or buffer. *F*, Platelet rich-plasma (PRP) from a healthy human donor was added with a collagen

(0.6  $\mu\text{g/ml}$ ) immediately followed by addition of different sources of LPS (1  $\mu\text{g/ml}$ ) or buffer to induce platelet aggregation.



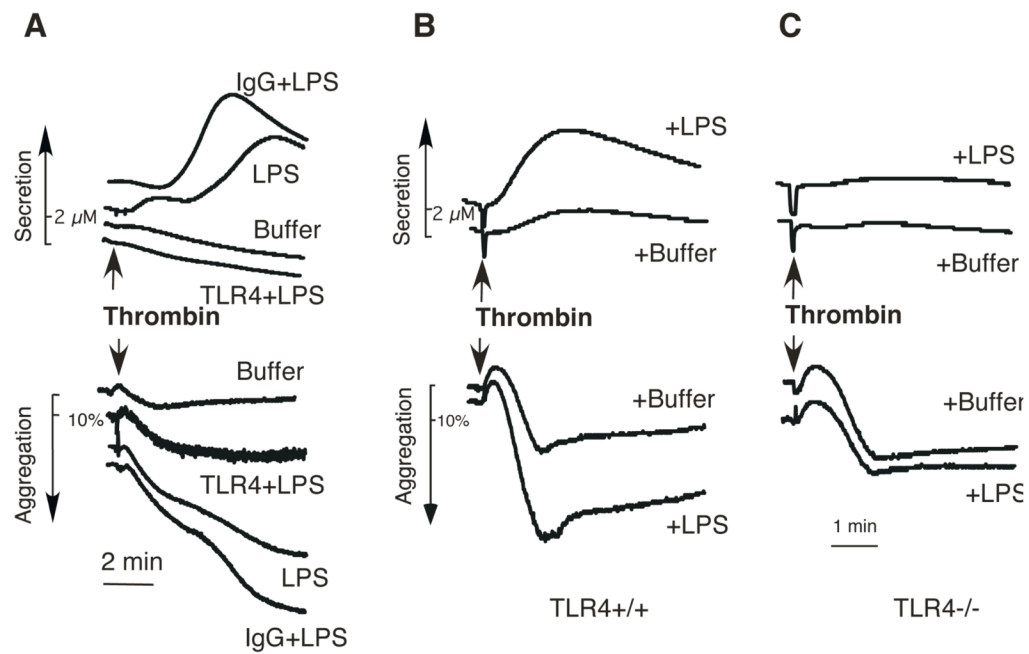
**Figure 2.**

LPS stimulates platelet secretion. *A*, Washed human platelets were incubated with 1  $\mu\text{g}/\text{ml}$  of LPS at 22  $^{\circ}\text{C}$  for 10 min. The release of ATP into the platelet supernatant was determined by luciferin/luciferase assay. *B* and *C*, Washed human platelets were incubated with LPS (1  $\mu\text{g}/\text{ml}$ ) or thrombin (0.025 U/ml) in the presence of a monoclonal anti-human P-selectin antibody, SZ51, for 30 min at room temperature. After washing, platelets were further incubated with a FITC-conjugated anti-mouse IgG. Expression of P-selectin was analyzed by flow cytometry. Quantitative results from three experiments are expressed as the P-selectin expression index (fluorescence intensity (mean) of platelets stimulated with an agonist/fluorescence intensity (mean) of unstimulated platelets) (*B*). Data from a representative experiment are shown in panel *C*.



**Figure 3.**

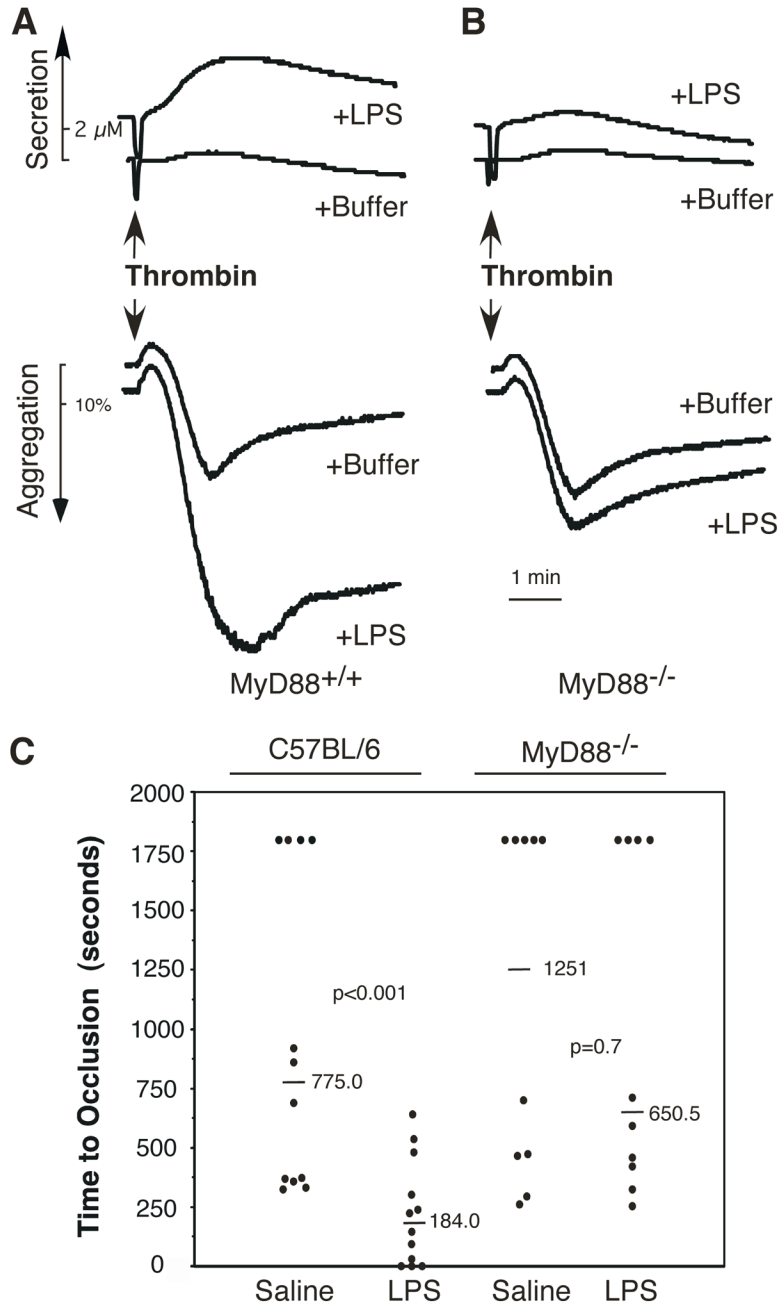
Expression of LPS receptor components in platelets. *A*, Washed human platelets (1 × 10<sup>9</sup>/ml) were solubilized in 1 × SDS-PAGE sample buffer. TLR4 was detected by immunoblotting with a rabbit anti-human TLR4 polyclonal antibody. β-actin was detected by immunoblotting with a mouse monoclonal antibody against β-actin as a positive control. *B*, *C*, and *D*. Washed human platelets were incubated with a rabbit anti-human TLR4 polyclonal antibody (*B*), or incubated with a mouse monoclonal antibody, which recognizes the human TLR4/MD2 complex (*C*), or with a mouse anti-human CD14 monoclonal antibody (*D*). The platelets were alternatively incubated with rabbit IgG or mouse IgG as controls. Expression of TLR4, TLR4/MD2 complex, and CD14 was analyzed by flow cytometry. *E*, Washed human platelets or various concentrations of WBCs from the same donor, were solubilized in 1 × SDS-PAGE sample buffer. MyD88 was detected by immunoblotting with a rabbit anti-human MyD88 polyclonal antibody. β-actin was detected by immunoblotting with a mouse monoclonal antibody against β-actin as a positive control.



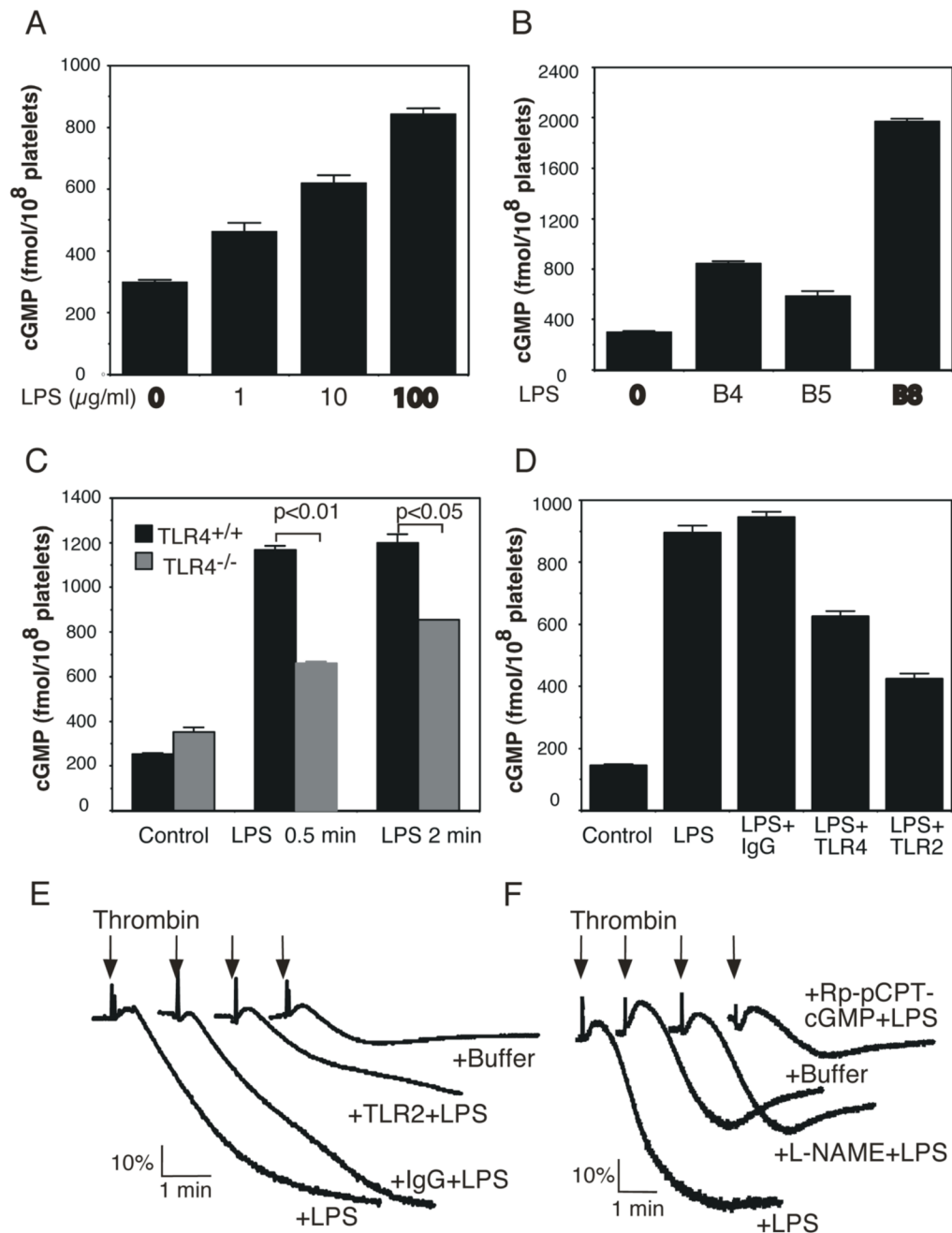
**Figure 4.**

LPS potentiates platelet activation via a TLR4-dependent signaling pathway. *A*, Washed human platelets were pre-incubated with a mouse monoclonal anti-human TLR4 blocking antibody (10  $\mu\text{g}/\text{ml}$ ) or control mouse IgG at 37°C for 5 min, and then exposed to a subthreshold concentration of thrombin in the presence of LPS (0111:B4, 10  $\mu\text{g}/\text{ml}$ ) or buffer. *B* and *C*, Washed platelets from wild type mice (C57BL/10J) (*B*) or TLR4 deficient mice (*C*) were exposed to low dose thrombin in the presence or absence of LPS (0111:B4, 10  $\mu\text{g}/\text{ml}$ ) to induce platelet secretion and aggregation.





**Figure 5.** The role of MyD88 in LPS-promoted platelet activation and thrombus formation. *A* and *B*, Washed platelets (resuspended in Tyrode’s buffer at  $3 \times 10^8$ /ml) from wild type (*A*) or MyD88 deficient mice (*B*) were added with low dose thrombin, followed by addition of LPS (0111:B4, 5  $\mu$ g/ml) and incubated in the aggregometer. *C*, FeCl<sub>3</sub>-induced carotid artery injury was performed and time to occlusive thrombosis recorded as described under *Materials and Methods*. LPS or saline was injected into fundus oculi of the C57BL/6 or MyD88 deficient mice 1 min after the initiation of carotid artery injury. The occlusion time of each mouse is shown as circles. The bars represent the median occlusion time.



**Figure 6.**

LPS-induced platelet activation involves the cGMP/PKG pathway. *A*, Washed human platelets were incubated with various concentrations of LPS (0111:B4) at 22 °C for 2 min. cGMP concentrations were determined using a cGMP enzyme immunoassay kit. The reaction was repeated three times using the platelets from the same donor. Results are expressed as mean  $\pm$  standard error. Statistical significance between groups was determined by t test ( $p < 0.05$  for LPS 1  $\mu\text{g/ml}$ , and  $< 0.001$  for LPS 10 or 100  $\mu\text{g/ml}$ ). The data shown here are representatives of at least three experiments from different donors. *B*, Washed human platelets were incubated with different sources of LPS (0111:B4 (B4), 055:B5 (B5), or 0127:B8 (B8)) 100  $\mu\text{g/ml}$  at 22 °C for 2 min. *C*, Washed platelets from TLR4 deficient mice or wild type controls were

incubated with LPS (0127:B8) (100 µg/ml) for 0.5 or 2 min. *D*, Washed human platelets were pre-incubated with mouse IgG, or mouse anti-TLR4 or TLR2 monoclonal antibodies (10 µg/ml) for 5 min at room temperature, and then incubated with LPS (0111:B4) (100 µg/ml) for 2 min. Statistical significance between groups was determined by t test ( $p < 0.05$  for TLR4 or TLR2 antibody treated platelets, compared to IgG-treated platelets). *E*, Washed human platelets were pre-incubated with mouse IgG or an anti-TLR2 blocking antibody for 5 min at 37°C, and then exposed to a low concentration of thrombin in the presence of LPS (0111:B4, 10 µg/ml) or buffer. *F*, Washed human platelets were pre-incubated with the NOS inhibitor, L-NAME (1 mM), or the PKG inhibitor Rp-pCPT-cGMPS (200 µM), at 37°C for 5 min, and then exposed to a subthreshold concentration of thrombin in the presence of LPS (0111:B4, 10 µg/ml) or buffer to induce platelet aggregation.