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A role for platelet glycoprotein Ib-IX and effects of its inhibition in endotoxemia-induced thrombosis, thrombocytopenia and mortality

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Abstract

Objective—Poor prognosis of sepsis is associated with bacterial lipopolysaccharide (LPS)-induced intravascular inflammation, microvascular thrombosis, thrombocytopenia, and disseminated intravascular coagulation. Platelets are critical for thrombosis, and there have been increasing evidence of the importance of platelets in endotoxemia. The platelet adhesion receptor, the glycoprotein Ib-IX complex (GPIb-IX), mediates platelet adhesion to inflammatory vascular endothelium and exposed subendothelium. Thus, we have investigated the role of GPIb-IX in LPS-induced platelet adhesion, thrombosis and thrombocytopenia.

Approach and Results—LPS-induced mortality is significantly decreased in mice expressing a functionally deficient mutant of GPIbα. Furthermore, we have developed a micellar peptide inhibitor, MPαC, which selectively inhibits the VWF-binding function of GPIb-IX and GPIb-IX-mediated platelet adhesion under flow without affecting GPIb-IX-independent platelet activation. MPαC inhibits platelet adhesion to LPS-stimulated endothelial cells *in vitro* and alleviates LPS-induced thrombosis in glomeruli in mice. Importantly, MPαC reduces mortality in LPS-challenged

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mice, suggesting a protective effect of this inhibitor during endotoxemia. Interestingly, MPαC, but not the integrin antagonist, Integrilin, alleviated LPS-induced thrombocytopenia.

Conclusion—These data indicate an important role for the platelet adhesion receptor GPIb-IX in LPS-induced thrombosis and thrombocytopenia, and suggest the potential of targeting GPIb as an anti-platelet strategy in managing endotoxemia.

Sepsis is a life-threatening systemic inflammatory state induced by the entry of bacterial endotoxins (such as lipopolysaccharides, LPS) into the blood circulation (endotoxemia). Mortality in severe sepsis is often associated with LPS-induced intravascular inflammation and thrombosis in microvasculature, which leads to impaired microcirculation, multiple organ failure, disseminated intravascular coagulation, and circulation failure (septic shock)[1, 2]. It is known that in order to improve the survival probability of septic patients, it is necessary not only to treat the source of infection, but also to treat systemic inflammation and microvascular thrombosis induced by endotoxemia and to improve the microcirculation.

There are two interrelated mechanisms causing thrombosis: (1) the coagulation cascade that catalyzes conversion of soluble fibrinogen in blood into a clot, and (2) platelet adhesion and aggregation, which not only form platelet thrombi, but also facilitate coagulation and inflammation. An anti-coagulant agent, the activated protein C (commercial name Xigris), was studied in clinical trials for its efficacy in the management of microvascular thrombosis and inflammation in adult sepsis. However, significant adverse effect of bleeding and associated mortality out-weighted the beneficial effect of the drug[3, 4]. It remains unclear regarding the exact roles of platelets in LPS-induced inflammation and microvascular thrombosis and whether platelet adhesion and activation contributes to mortality in endotoxemia patients. However, there is increasing evidence suggesting the importance of platelets in endotoxemia: LPS sensitizes platelets to agonist stimulation[5], induces the accumulation of platelets in the blood vessels of certain organs such as the lung and liver[6, 7], and induces consumptive thrombocytopenia[6, 8, 9], which is associated with poor outcome of septic patients.

In the microcirculation, particularly arterioles wherein blood flows at relatively high shear rates, platelet adhesion to the vascular wall is dependent upon the interaction between GPIb-IX and its ligand, von Willebrand factor (VWF)[10–12]. GPIb-IX has also been shown to be important in venous thrombosis[13, 14]. GPIb-IX consists of GPIbα, GPIbβ, GPIX, and GPV[15]. The extracellular N-terminal region of GPIbα contains the binding sites for VWF and thrombin[15], whereas the cytoplasmic domain of GPIbα is linked to the actin cytoskeleton via filamin[16, 17]. The cytoplasmic domain of GPIb also interacts with 14-3-3ζ[18–20]. A major binding site for 14-3-3ζ is located in the C-terminal region of GPIbα[18, 21]. The binding of 14-3-3ζ to the C-terminus of GPIbα is important for regulating the VWF binding function of GPIb-IX and in thrombosis[22, 23]. In this study, we demonstrate that GPIb-IX plays an important role in the LPS-induced platelet adhesion to inflammatory endothelial cells *in vitro*, LPS-induced glomerular microvascular thrombosis *in vivo*, and in the mortality of LPS-challenged mice. We also show that an inhibitor of the ligand binding function of GPIb-IX, based on GPIbα C-terminal sequence, micellar MPαC, prevents LPS-induced thrombosis in microvasculature in glomeruli, ameliorates LPS-induced

thrombocytopenia, and decreases the mortality of LPS-challenged mice. Thus, GPIb-IX is a potentially important target for developing new drugs for the management of severe endotoxemia.

Materials and Methods

Materials and methods are detailed in the online supplement. Myristoylated peptides MPαC (C13H27CONH-SIRYSGHpSL) and MCsC (C13H27CONH-LSISYGSHR) were formulated as micelles with PEG_{2000}-DSPE (Avanti Polar Lipids Inc. Alabaster, AL), L-α-phosphatidylcholine (egg PC, Type XI-E, Sigma-Aldrich, St. Louis, MO) and peptides at a molar ratio of 45:5:1(in some experiments, 45:5:2) as previously described[24]. **T**he IL-4R/Ibα mice lack the gene for endogenous mouse GPIbα but instead express a fusion protein where the extracellular sequences of human GPIbα have been replaced by an extracellular domain from the interleukin-4 receptor[25]. Platelet adhesion to endothelial monolayers was assayed under shear stress introduced using a cone-plate rheometer (Rheostress 1, Thermo-HAAKE, Paramus, NY)[26, 27].

Results

The importance of GPIb-IX in LPS-induced mortality

We have used two approaches: a genetic approach and a selective inhibitor of GPIb-IX function to determine the importance of GPIb-IX in endotoxemia, and the potential of inhibition of GPIb-IX in management of endotoxemia. GPIb-IX deficient mice have significant thrombocytopenia and abnormally sized platelets, and thus cannot be used to dissect the consequence of defects in GPIb-IX function. Thus, we tested LPS-induced mortality in transgenic mice expressing a fusion protein of the IL-4 receptor extracellular domain and GPIbα transmembrane and cytoplasmic domain (IL-4R-Ibα). IL-4R-Ibα lacks the receptor function of GPIb-IX, but preserves the function of the cytoplasmic domain of GPIb to interact with the membrane skeleton, thus maintaining the platelet shape and ameliorating thrombocytopenia in GPIbα$^{-/-}$ mice[25, 28]. Compared with wild type mice, IL4-Ibα mice showed a significant reduced mortality when challenged with LPS, indicating that a functional GPIb-IX complex on the platelet surface is important in LPS-induced mortality in this mouse endotoxemia model (Fig. 1).

Micellar MPαC and its effects on GPIb-IX-dependent platelet function

To determine the role of GPIb-IX in human and mouse platelet adhesion and *in vivo* thrombosis during endotoxemia and to evaluate the potential of targeting GPIb, it is necessary to develop GPIb-IX inhibitors. However, a common problem in developing GPIb-IX inhibitors is that the GPIb extracellular domain-binding molecules and antibodies induce severe thrombocytopenia *in vivo*. To overcome this problem, we applied knowledge of the important role of the GPIbα cytoplasmic domain 14-3-3 binding site in GPIb-IX function, and developed a phosphotidylcholine/DSPE-PEG2000 micellar delivery system incorporating a myristoylated peptide, Myr-SIRYSGHpSL (MPαC), corresponding to the C-terminal 14-3-3ζ binding sequence of GPIbα, and a myristoylated scrambled control peptide (MCsC). The micellar formulation allows safe *in vivo* use of the highly hydrophobic MPαC

without requiring toxic solvents. Indeed, no noticeable adverse effect was observed in mice following retro-orbital injection of micellar MPαC and control micellar MCsC, which have similar pharmacokinetics in the circulation (Supplemental Fig. I). To examine whether micellar MPαC inhibited 14-3-3ζ binding to GPIb-IX, micellar MPαC and MCsC were preincubated with platelets. Platelets were then solubilized and immunoprecipitated with an anti-GPIbα antibody. Micellar MPαC, but not micellar MCsC, inhibited the co-immunoprecipitation of GPIb-IX and 14-3-3ζ by ~80% (Fig. 2A, 2B), indicating that micellar MPαC is effective in blocking 14-3-3ζ binding to GPIb-IX.

To determine whether micellar MPαC affects GPIb-dependent platelet function, human platelet rich-plasma was pre-treated with micellar MPαC or control micellar MCsC. VWF/GPIb-IX-dependent platelet agglutination/aggregation was induced by adding ristocetin, which allows soluble VWF binding to GPIb-IX. Micellar MPαC dose-dependently inhibited ristocetin-induced human platelet aggregation (Fig. 2C). In contrast to the inhibitory effect of MPαC on VWF/GPIb-IX-mediated platelet aggregation, micellar MPαC treatment of platelet-rich plasma had no significant effect on platelet aggregation induced by the GPIb-IX-independent agonists ADP, collagen, and U46619 (a thromboxane A_2 analogue) (Fig. 2D). Thus, micellar MPαC selectively inhibits GPIb-IX-dependent platelet function without inhibiting general platelet activation signaling.

The effect of micellar MPαC on arterial thrombosis *in vivo*

GPIb-IX is known to play an important role in arterial thrombosis[29]. Thus, we evaluated the *in vivo* effect of micellar MPαC on arterial thrombosis using the ferric chloride ($FeCl_3$)-induced mouse carotid artery thrombosis model. Retro-orbital injection of micellar MPαC significantly ($P<0.01$) delayed $FeCl_3$-induced carotid artery occlusive thrombosis compared to the control (Fig. 2E), indicating that micellar MPαC is an effective inhibitor of arterial thrombosis *in vivo*.

LPS-induced GPIb-IX-dependent platelet adhesion to vascular endothelial cells and the inhibitory effect of MPαC

Although LPS is known to promote injury-induced thrombosis [5, 30], it is unclear how LPS alone induces thrombosis in endotoxemia. The role of platelets in thrombosis under high shear rate flow conditions allows us to hypothesize that GPIb-IX-dependent platelet adhesion to the vascular endothelium may play a role in initiating LPS-induced microvascular thrombosis. To test this hypothesis, we investigated whether LPS can directly induce platelet adhesion to vascular endothelium under high shear rate conditions. A confluent monolayer of human umbilical vein endothelial cells (HUVECs) was pretreated with LPS before exposure to washed human platelets under flow at 800 s^{-1} shear rate. As expected, platelets poorly adhered to unstimulated endothelial cell surfaces (Fig.3, A, B, and C). LPS treatment of endothelial cells, however, slowly and transiently induced platelet adhesion to HUVEC cells, with a maximal effect at 1 hour (Fig. 3A). Treatment of platelets with micellar MPαC, but not the control micellar MCsC, inhibited LPS-induced platelet adhesion (Fig. 3, B and C). Similarly, an anti-GPIbα monoclonal antibody, LJ-P3, also inhibited platelet adhesion to LPS-stimulated HUVEC cells (Fig. 3D). Importantly, the presence of isolated human leukocytes greatly enhanced and accelerated LPS-induced

platelet adhesion to endothelial cells, which was also inhibited by MPαC (Fig. 3E). These data suggest that LPS induces platelet adhesion to the vascular endothelium by leukocyte-dependent and independent mechanisms. These data also suggest that LPS-induced platelet adhesion to endothelial cells requires GPIb-IX function, and that MPαC antagonizes LPS-induced platelet adhesion to the vascular endothelium.

The effect of MPαC in LPS-induced microvascular thrombosis in an endotoxemia mouse model

To investigate whether MPαC attenuates LPS-induced microvascular thrombosis, we examined the effect of micellar MPαC on LPS-induced microvascular thrombosis in the kidney glomeruli of C57/BL mice. Intraperitoneal injection of LPS induced microvascular thrombosis in kidney glomeruli, as indicated by Mallory's phosphotungstic acid hematoxylin staining of platelets and fibrin deposition (Fig. 4A). LPS-induced microvascular thrombosis was significantly reduced in micellar MPαC-treated mice, as compared with the MCsC micelles (Fig 4A). Similar results were also obtained with an immunohistochemical staining of microvascular thrombi with an anti-platelet integrin α_{IIb} antibody (Fig. 4B). Thus, our data indicate that GPIb-IX plays a critical role in LPS-induced microvascular thrombosis *in vivo*, and that disruption of GPIb-dependent platelet function by MPαC is effective in preventing LPS-induced microvascular thrombosis.

The effect of micellar MPαC and Integrilin on survival rate in LPS-challenged mice

The effect of MPαC in alleviating LPS-induced platelet-endothelial cell adhesion and microvascular thrombosis suggests the potential to use MPαC in the management of endotoxemia. To determine the effect of MPαC on endotoxemic mice, we assessed the mortality in mice challenged with LPS. Injection of a high concentration of LPS (22 mg/kg) caused 70% mortality in control mice within 48 hours (Fig. 5A). Injection of the control micellar MCsC had no significant effect on LPS-induced mortality (Fig. 5A). In comparison, a single dosage of micellar MPαC injection before LPS challenge significantly reduced LPS-induced mortality to ~30% (P=0.02) (Fig. 5A), suggesting the important role of GPIb-IX-dependent platelet adhesion in the process. Interestingly, injection of MPαC 1 hour after LPS challenge also significantly reduced mortality in comparison with scrambled control MCsC, suggesting a possible therapeutic potential (Fig. 5B). Consistent with the importance of platelets in LPS-induced mortality, injection of an integrin inhibitor, Integrilin, similarly showed a trend in reducing the LPS-induced mortality (Fig. 5C), indicating a role for the platelet-dependent microvascular thrombosis in the grave consequences of endotoxemia, and the beneficial effect of anti-platelet therapy. While the effects of MPαC and Integrilin in reducing mortality are similar, injection of micellar MPαC to mice only mildly prolonged the tail bleeding time (Fig. 5D). In contrast, Integrilin caused dramatically prolonged tail bleeding time in mice (Fig. 5E). These findings indicate that GPIb-IX-dependent microvascular thrombosis contributes to mortality in sepsis. Our results also suggest that micellar MPαC has the potential to be developed into an effective agent in reducing LPS-induced mortality with only a very mild tendency towards bleeding side effect.

The effect of micellar MPαC on LPS-induced thrombocytopenia

It is known that severe sepsis in patients or injection of LPS in mice is associated with thrombocytopenia. Thus, we investigated whether MPαC or Integrilin affected LPS-induced changes in circulating platelet counts. Intraperitoneal injection of 12 mg/kg LPS caused a platelet count decrease of ~ 50% 4 hours following LPS injection (Fig. 6A). A single injection of micellar MPαC, partially, but significantly mitigated LPS-induced thrombocytopenia (Fig. 6A). In contrast to the significant effect of MPαC, injection of 5 mg/kg Integrilin had no effect on LPS-induced thrombocytopenia (Fig. 6B) despite its significant effect in prolonging bleeding time (Fig. 5E) and in reducing mortality (Fig. 5C). To exclude the possibility that MPαC affected platelet counts independent of LPS, we also examined platelet counts in healthy mice treated with micellar MPαC or the control micellar peptide MCsC. MPαC did not induce changes in platelet counts without LPS injection (Fig. 6C). These data indicate that LPS-induced thrombocytopenia involves GPIb-IX function but not that of integrin $\alpha_{IIb}\beta_3$. Thus, LPS-induced thrombocytopenia may not be a direct consequence of thrombus formation, but a consequence of GPIb-IX-dependent platelet clearance. These data also indicate that micellar MPαC is effective in mitigating endotoxemia-induced thrombocytopenia.

Discussion

Our data indicate that GPIb-IX plays an important role in platelet adhesion to LPS-stimulated vascular endothelial cells, microvascular thrombosis and LPS-induced thrombocytopenia and mortality. In addition, we show that a GPIb inhibitor based on the C-terminal sequence of the cytoplasmic domain of GPIbα is effective in ameliorating LPS-induced microvascular thrombosis, thrombocytopenia, and mortality *in vivo*. These data suggest a potential new strategy for the management of endotoxemia-induced microvascular thrombosis and consequent multiple organ failure, and for improving the survival rate of endotoxemia patients.

It is currently recognized that poor prognosis of endotoxemia in sepsis is associated with systemic microvascular thrombosis and disseminated intravascular coagulation[1, 2]. However, the mechanism that initiates microvascular thrombosis in endotoxemia remains unclear. The current concept emphasizes the initiation of the coagulation cascade. Thus, the clinical anti-thrombotic treatment of severe sepsis is limited to anti-coagulant agents such as recombinant activated protein C[3]. Our study suggests that platelet adhesion, particularly GPIb-IX-dependent platelet adhesion, is important in LPS-induced microvascular thrombus formation. Thus, inhibitors of platelet adhesion appear to reduce LPS-induced platelet-endothelial cell interaction, microvascular thrombosis and mortality of LPS-treated mice. These data are consistent with the concept that platelet adhesion, particularly GPIb-IX-dependent platelet adhesion, is important for thrombosis in microcirculation[10], and suggest the potential for GPIb-IX inhibitors as anti-thrombotics in the management of endotoxemia.

The importance of platelet GPIb-IX in endotoxemia and LPS-induced microvascular thrombosis is supported by experiments using a combination of genetic manipulation and pharmacological inhibitors in mouse models: A major challenge in studying endotoxemia and sepsis are the animal endotoxemia models may not precisely replicate the wide range of

pathophysiologic events occurring in humans. Thus, results obtained with mouse or other animal models, while very valuable as a guide and theoretical basis, need to be carefully evaluated in treating human endotoxemia. A more extreme viewpoint on this issue was published in a recent report proposing bypassing animal models for the reason that the induced gene expression patterns are different between humans and mice under inflammatory conditions[31]. However, that report did not compare gene expression or consequent functional responses between human and mouse megakaryocytes and platelets in their models. More importantly, the data from that publication are not sufficient to exclude the similarities between humans and mice in inflammatory response. For example, comparison of induced gene expression patterns is unlikely to reveal the importance of specific molecular mechanisms of inflammation that may be similar between humans and mice. Furthermore, although different in overall pattern, there is about 47–61% similarity in gene changes between human and mice in response to inflammatory stimuli as shown in that study[31]. It is possible that these similarly changed genes may represent those specific molecules that play key roles in inflammatory responses. It is important to note that conducting severe endotoxemia models similar to the stringently controlled animal experiments is unethical to perform in humans. Furthermore, it is well-established that human, mice, and other animals share very similar molecular mechanisms and genetic bases in physiology and pathology including thrombosis and inflammation. Thus, animal models are often the only available means for the medical community to study severe endotoxemia (and many other severe diseases) in order to obtain knowledge regarding the molecular mechanisms and discover drug targets for the treatment of human diseases. This approach has proven valuable. Abandoning of animal models by over-emphasis of the difference between humans and animals will prove detrimental to our progress in understanding and development of new treatment for severe human diseases.

To be able to further test whether GPIb-IX-mediated platelet adhesion and activation is important in severe endotoxemia in humans, it is necessary to develop new drugs that can interfere with GPIb-IX function *in vivo* in endotoxemic patients. In this regard, MPαC is a new type of inhibitor that selectively interferes with GPIb-IX function without affecting GPIb-IX-independent platelet activation pathways. Unlike the GPIb-IX antagonists currently under development, MPαC acts on the cytoplasmic domain of GPIbα and does not induce thrombocytopenia. In fact, a major novelty of this study is the discovery that MPαC alleviates thrombocytopenia induced by LPS. Micellar MPαC represents not only a potential new strategy for the management of endotoxemia-induced microvascular thrombosis and potentially for improving the survival rate of sepsis patients, but also the only inhibitor that inhibits GPIb-IX-dependent platelet clearance. Interestingly, in hemolytic uremic syndrome, bacterial shiga toxin has been known to induce thrombotic microangiopathy and consumptive thrombocytopenia[32]. In inherited thrombotic thrombocytopenic purpura, VWF-GPIb interaction also causes microvascular thrombosis and thrombocytopenia. Thus, it would be interesting to further investigate whether MPαC can also be used in the treatment of hemolytic uremic syndrome and other types of thrombotic microangiopathy that involves VWF binding to GPIb-IX. Nevertheless, although we conclude that MPαC exerts its effects mainly through inhibiting 14-3-3 binding to GPIb, we do not totally exclude the possibility of additional effects of MPαC on other functions of 14-3-3. However, such additional

effects of MPαC, if present, are also likely to involve the GPIb-IX signaling pathway because MPαC does not significantly affect GPIb-IX-independent platelet activation and mimics the anti-thrombotic effect seen in GPIb-IX functional deficiency.

Our data indicate that endothelial cell exposure to LPS alone is sufficient to induce a slow endothelial cell response that allows GPIb-IX-dependent platelet adhesion. However, LPS rapidly and potently induces platelet adhesion to endothelial cells in the presence of peripheral blood leukocytes, indicating that inflammatory mediators released by LPS-stimulated leukocytes induce rapid exposure of GPIb-IX ligands on endothelial cell surfaces. Large quantities of VWF are stored in endothelial Weibel-Palade bodies and become exposed on the endothelial cell surface upon stimulation by inflammatory mediators[33, 34]. It has been reported that platelets adhere to inflammatory mediator (histamine)-activated endothelial cells and form a “beads-on-a-string” like structure due to exposure of ultra-large VWF multimers on the endothelial surface[34]. Thus, it is likely that LPS and inflammatory mediator-induced exposure of endothelial-bound VWF is important in mediating GPIb-IX-dependent platelet adhesion. In this respect, suppression of the VWF-cleaving activity of ADAMTS13 has been reported in patients suffering from sepsis [35]. Also, increased VWF levels and the presence of more active ultra-large VWF in plasma have been found in severe sepsis and septic shock patients[36]. In a sepsis model, VWF-deficient mice lived significantly longer than wild type mice[37], although an earlier report by some of the coauthors of that work did not show significant difference between VWF-deficient mice and wild type controls following LPS challenge[38]. It is also possible that the role of GPIb-IX may be mediated by its binding to endothelial P-selectin [39] and endothelial-bound coagulation factors (such as thrombin)[40] or through leukocyte or endothelial integrins[41, 42], although there have been studies challenging the role of P-selectin and integrins in platelet string formation on endothelial cells[43]. It is interesting to further investigate the mechanism by which GPIb-IX mediates microvascular thrombosis in endotoxemia and whether an anti-platelet therapy will have an advantage over anti-VWF therapy in treating platelet-dependent microvascular thrombosis.

Our data not only indicate that MPαC inhibits LPS-induced microvascular thrombosis, but also that it alleviates thrombocytopenia induced by LPS. Consumptive thrombocytopenia in sepsis and endotoxemia is associated with poor prognosis[1, 2]. Apart from the deposition of activated platelets in microvascular thrombi, thrombocytopenia is mainly caused by the clearance of LPS-stimulated platelets from the circulation by the liver and spleen[44, 45]. Interestingly, a recent study suggests that clearance of both VWF and platelets involves Ashwell receptors in hepatocytes[44, 46]. Thus, it is tempting to speculate LPS-induced consumptive thrombocytopenia is partially dependent upon Ashwell-receptor recognition of platelet-bound VWF or Ashwell receptor recognition of a VWF-bound conformational state of GPIb-IX. In this respect, it is worth noting that MPαC is unique as a GPIb-IX inhibitor, because it inhibits GPIb-IX function by competitively blocking 14-3-3 binding to GPIb, thus restricting GPIb-IX to a conformational state that is unable to bind VWF. Clearly, this conformation of GPIb-IX also prevents clearance of platelets from the circulation. In summary, this study shows that GPIb-IX plays an important role in severe endotoxemia, and an inhibitor of GPIb-IX function alleviates LPS-induced platelet adhesion, microvascular

thrombosis, and consumptive thrombocytopenia which may potentially be effective in the management of endotoxemia in sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LPS	lipopolysaccharides
GPIb-IX	the glycoprotein Ib-IX complex
VWF	von Willebrand factor
HUVEC	human umbilical vein endothelial cells.

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Significance

This work demonstrates the importance of the platelet adhesion receptor, GPIb-IX, in endotoxemia, and the *in vivo* effects of an inhibitor of GPIb-IX interaction with its intracellular binding partner, 14-3-3 protein, in reducing endotoxemia-induced microvascular thrombosis in glomeruli, thrombocytopenia and mortality. These results provide a new mechanism for the endotoxemia-induced microvascular thrombosis and thrombocytopenia, and suggest potential for the GPIb-IX inhibitor in managing endotoxemia.

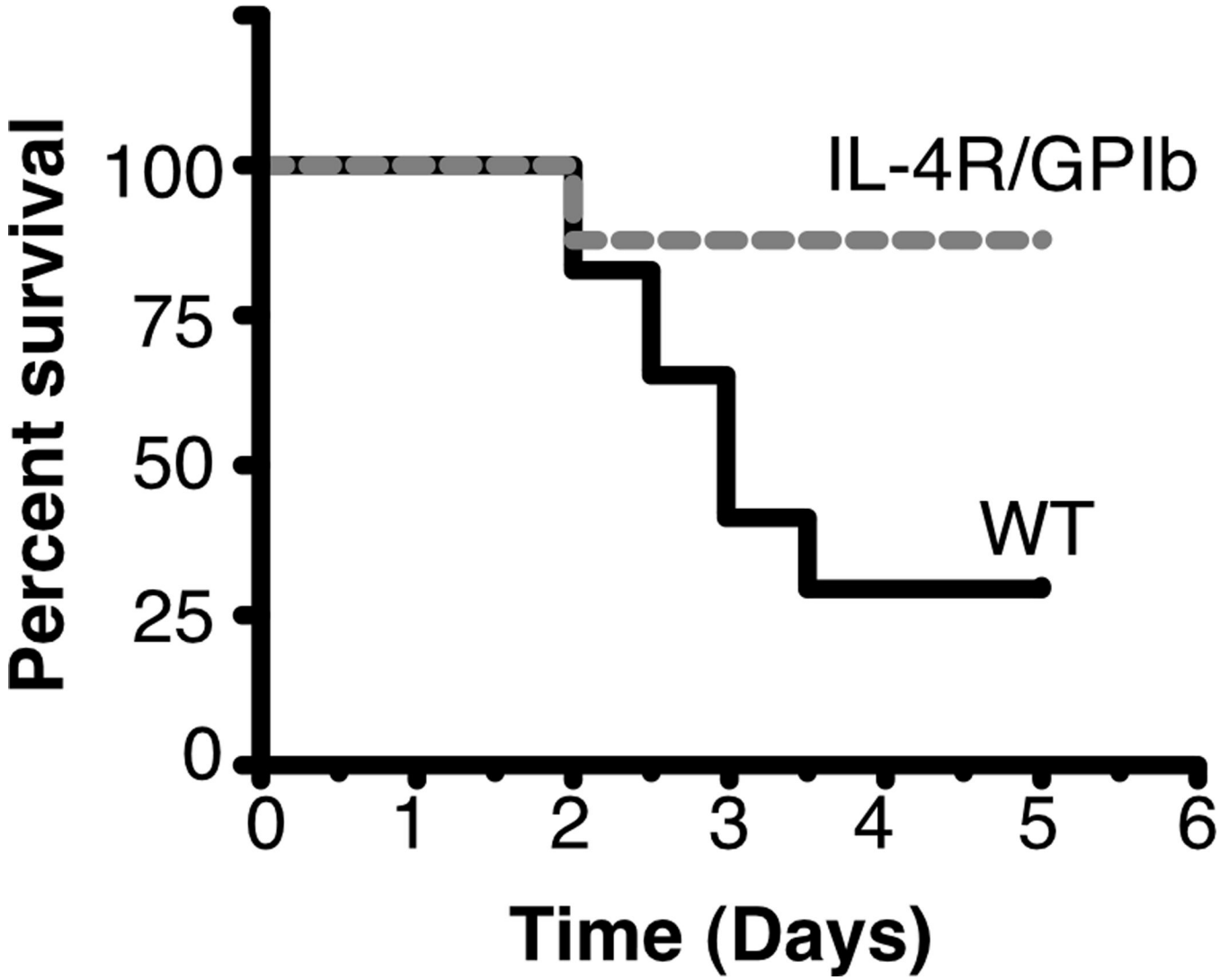

Figure 1. The importance of platelet GPIb-IX in LPS-induced mortality in a mouse endotoxemia model

The wild type (WT, n=17) and IL-4R/Ibα (n=16) mice were challenged by intraperitoneal injection of LPS (100 μg) and their mortality monitored. Mouse survival rate was estimated using Kaplan-Meier plot. Logrank test shows that the difference between WT and IL-4R/Ibα is highly significant ($p<0.002$).

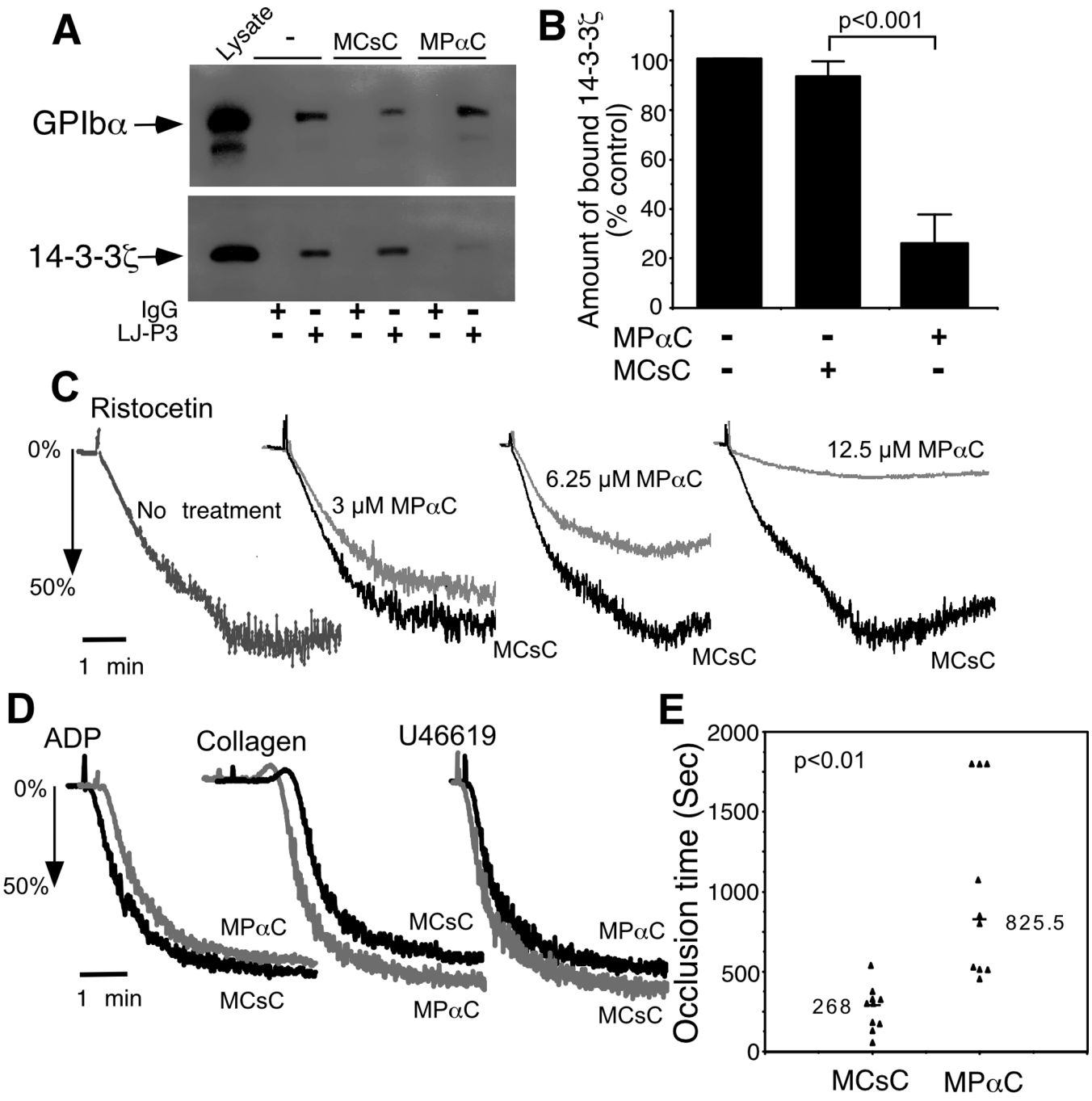

Figure 2. Selective inhibition of GPIb-IX-dependent platelet function and arterial thrombosis by micellar MPαC

(A) Platelets were preincubated with MPαC or control MCsC, solubilized and then immunoprecipitated with an anti-GPIbα antibody (LJ-P3). Immunoprecipitates were immunoblotted with anti-GPIbα and anti-14-3-3ζ antibodies. (B) GPIb-bound 14-3-3ζ was quantified by measuring immunoblot band intensity (mean ± SD, 3 experiments, t-test). (C) Human platelet-rich plasma (PRP) was preincubated with or without increasing concentrations of the MPαC or control peptide for 15 minutes, and stimulated with ristocetin (1.2 mg/ml) to induce platelet aggregation. (D) Human PRP was preincubated with 12.5 μM

MPαC or MCsC for 15 minutes and stimulated with ADP (5 μM), collagen (2 μg/ml) and the thromboxane A_2 analog, U46619 (1.6 μM) (shown in the figure are representative aggregation curves from 3 independent experiments using blood from 3 donors). (E) C57BL/6J mice were retro-orbitally injected with MPαC or control peptide (2 μmol/kg) 15 minutes before the carotid artery injury by ferric chloride (10%). Blood flow was recorded until vessel occlusion. The experiment was repeated 10 times using 10 mice for each group (MPαC and MCsC, $P < 0.01$, t-test with Welch correction). Each triangle represents the occlusion time of each mouse.

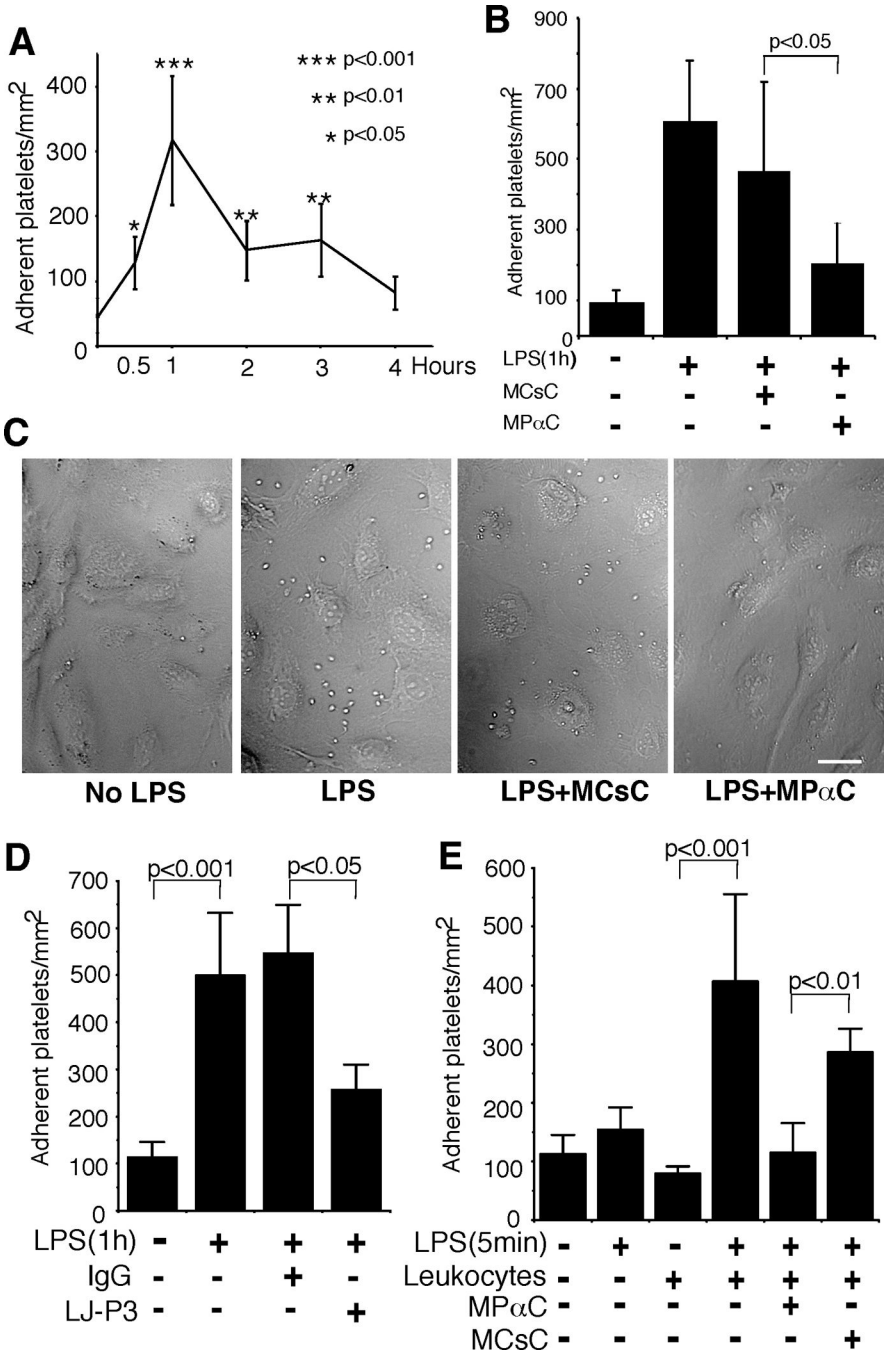

Figure 3. The effect of MPαC and an anti-GPIb antibody on platelet adhesion to LPS stimulated endothelial cells under shear stress

(A) HUVEC monolayers were preincubated without (0 hour) or with LPS (20 μM) for various lengths of time. Washed platelets ($3X10^8$/ml) were then allowed to adhere to these HUVECs under constant shear rate ($800s^{-1}$) for 5 minutes. (B) HUVEC monolayers were stimulated without or with LPS for 1 hour. Platelets ($3X10^8$/ml) were preincubated with micellar peptides (50 μM) for 30 minutes, and then allowed to adhere to HUVECs under a constant shear rate ($800s^{-1}$) for 5 minutes. (C) Representative pictures from (B) Size of the scale bar = 25 μm. (D) Platelets were preincubated without or with 10 μg/ml of an inhibitory

anti-GPIbα antibody LJ-P3 or IgG control for 30 minutes, and then allowed to adhere to LPS-stimulated or control HUVEC monolayers as in (B). (E) HUVECs were preincubated with or without LPS for 5 minutes. Platelets were preincubated with or without 50 μM MPαC or MCsC and then allowed to adhere to HUVECs under shear stress for 5 minutes with or without simultaneous addition of peripheral blood leukocytes. Platelets directly adhering to the HUVEC monolayer in each assay were quantified (mean ± SD, 3 experiments). Statistic significance was determined using nonparametric ANOVA in all assays in this figure.

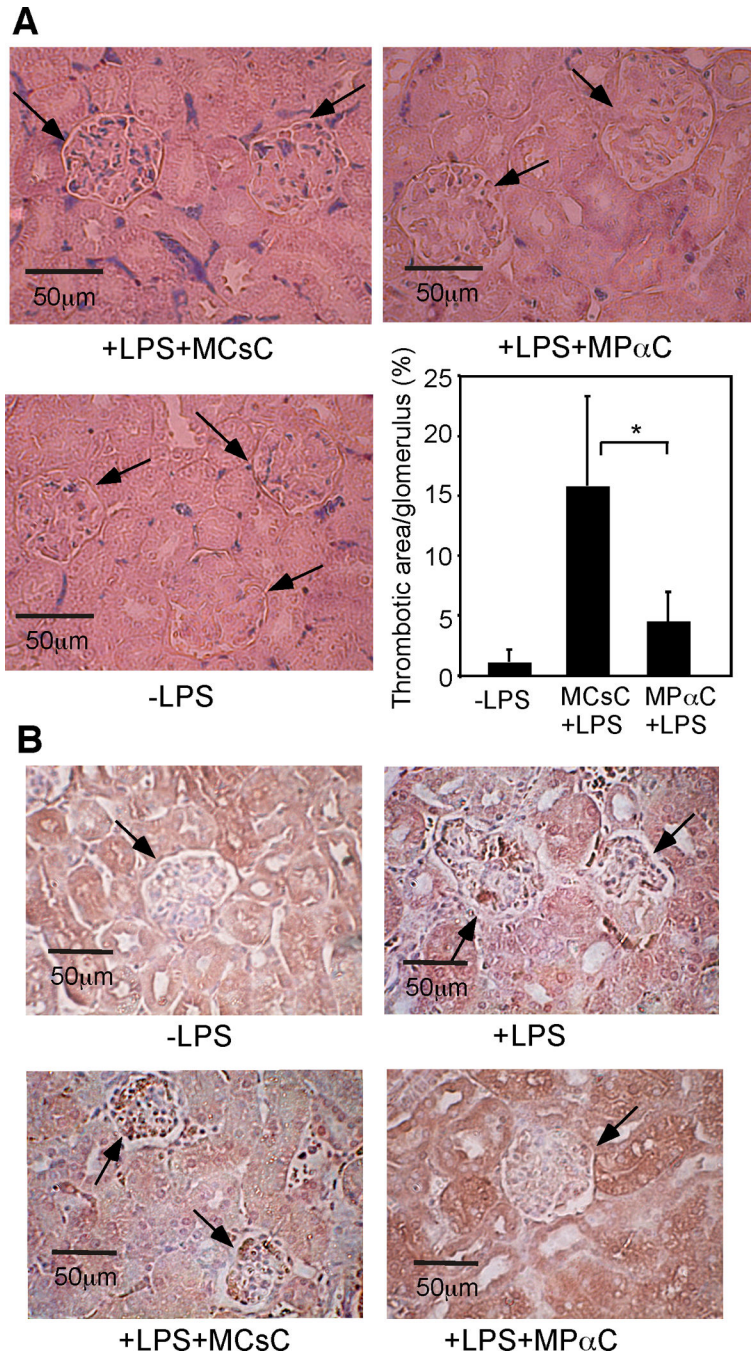

Figure 4. The effect of MPαC on LPS induced microvascular thrombosis in kidney glomeruli

(A) C57BL mice were retro-orbitally injected with 2 μmol/kg MPαC or control MCsC prior to intraperitoneal injection of LPS (22 mg/kg). Kidney sections from peptide treated- or control mice were stained with phosphotungstic acid hematoxylin to show fibrin and platelets in the glomeruli. The experiment was repeated 6 times using 6 different mice for each group. Bars indicate the average (±SD) of the percentage of phosphotungstic acid hematoxylin stained area per glomerulus (50 random glomeruli from 6 mice/group) (*P<0.001, t-test with Welch correction). (B) Kidney sections from MPαC- or MCsC-

treated mice or control mice were also immunostained with an anti-integrin α_{IIb} monoclonal antibody to show the platelet-rich thrombi. Shown in the figure is a typical image from 6 experiments (6 mice). Arrows point to glomeruli in which microvascular thrombi are concentrated.

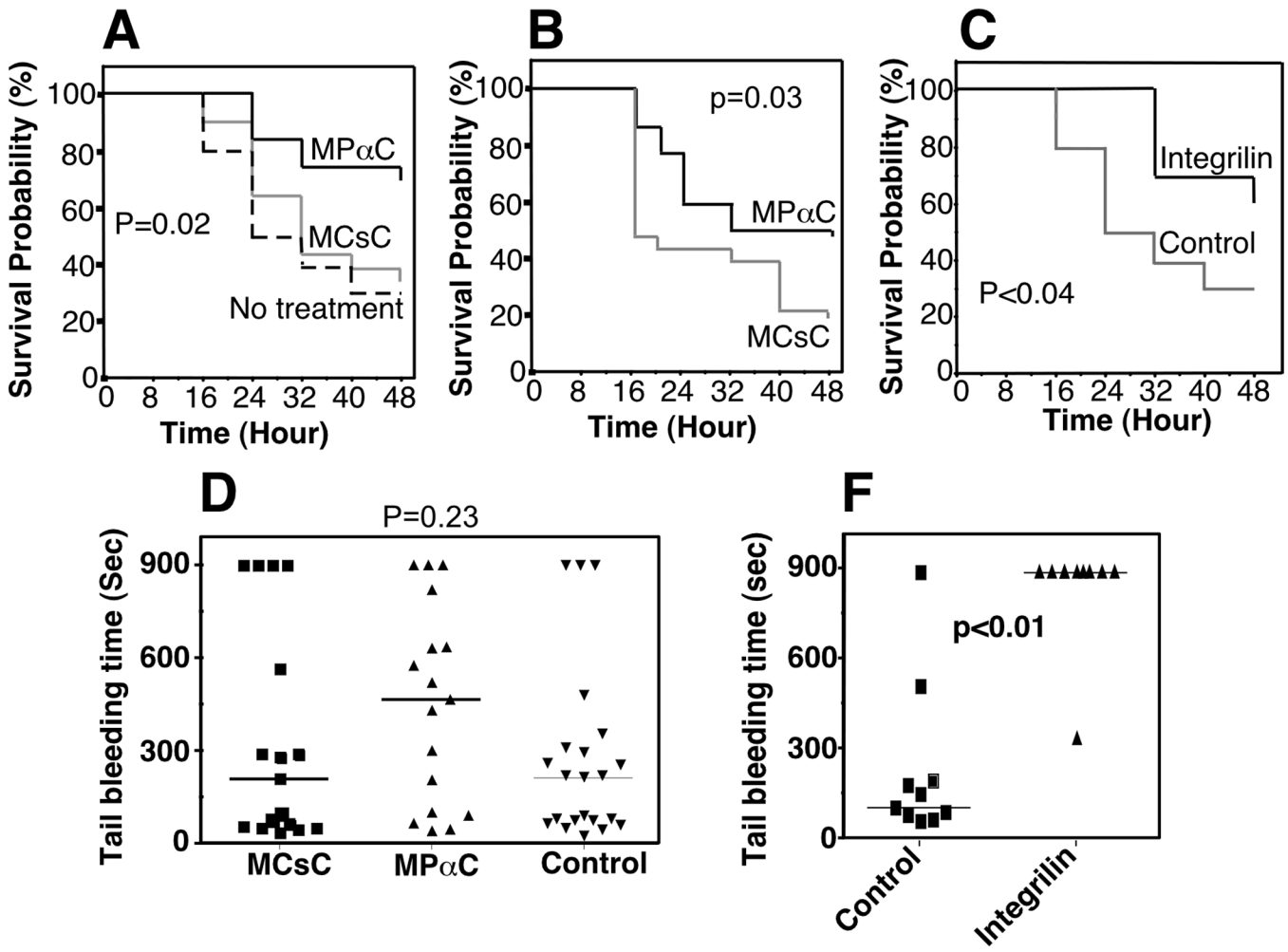

Figure 5. The effect of MPαC on the survival probability of LPS-challenged mice

(A) C57BL/6 mice retro-orbitally injected with micellar MPαC (2 μmol/kg) (n=20) or MCsC (n=20), and mice without micellar peptide injection (No treatment) were intraperitoneally injected with LPS (22 mg/kg). Mouse survival rate was estimated using the Kaplan-Meier plot (20 mice/group, p=0.02, MPαC vs MCsC). (B). C57BL/6 mice were first injected with LPS, one hour prior to injection of MPαC (10μmol/kg) (n=10) or MCsC control (n=10) micelles. Survival rates at different time points were recorded as in (A). (C) C57BL/6 mice were retro-orbitally injected with or without Integrilin (5 mg/kg), prior to intraperitoneal LPS injection. Mouse survival rate was estimated using the Kaplan-Meier plot (10 mice/group). (D) C57BL/6 mice were retro-orbitally injected with micellar 2 μmol/kg MPαC or control MCsC 15 minutes before transection of the tail. The time to the cessation of tail bleeding was recorded and shown as closed triangles. The experiment was repeated using multiple mice as indicated for each group (MCsC, n=16, MPαC, n=17, no treatment n=22, P=0.23, Kruskal-Wallis non-parametric ANOVA, median are shown as bars). (E) C57BL/6 mice were retro-orbitally injected with Integrilin (Integrilin,5 mg/kg) (n=10) or saline (n=10) 5 minutes before tail bleeding time analysis (*P<0.01, Mann-Whitney test, median value are indicated as bars).

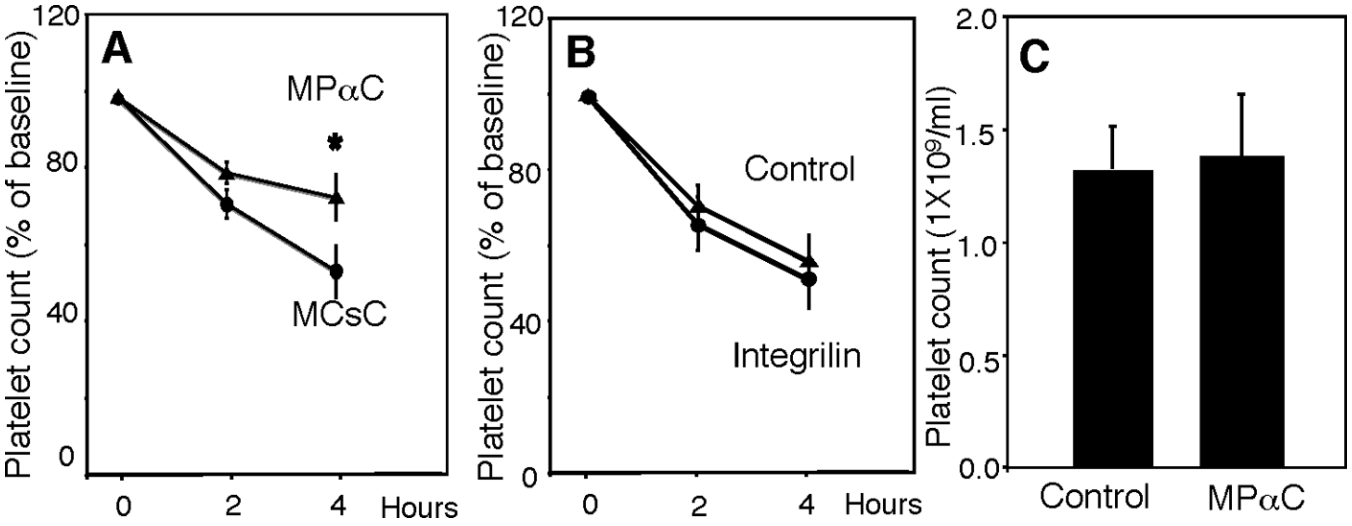

Figure 6. The effect of MPαC on LPS-induced thrombocytopenia

(A) C57BL/6 mice were retro-orbitally injected with 2 μmol/kg MPαC or control MCsC prior to intraperitoneal (i.p.) injection of LPS (12 mg/kg) (10 mice/group). Blood platelet counts were obtained before and after LPS challenge for 2 and 4 hours. The experiment was repeated 15 times using 15 different mice. The percentage of platelets remaining in the peripheral blood is shown (mean±SD, * $P<0.05$, n=15/group). (B) Relative platelet counts of C57BL/6 mice retro-orbitally injected with Integrilin (5 mg/kg) or saline before, 2 hours, and 4 hours after challenge with LPS (12 mg/kg) i.p. injection (repeated using 10 mice/group, mean±SD). (C) Platelet counts of C57BL/6 mice before and 4 hours after retro-orbitally injected with MPαC (2 μmol/kg) or vehicle (buffer) only (repeated using 10 mice/group, mean±SD).