Role for Platelet Glycoprotein Ib-IX and Effects of its Inhibition in Endotoxemia-Induced Thrombosis, Thrombocytopenia, and Mortality

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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 has 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, MPtC (C13H27CONH-SIRYSGHpSL), which selectively inhibits the von Willebrand factor -binding function of GPIb-IX and GPIb-IX–mediated platelet adhesion under flow without affecting GPIb-IX–independent platelet activation. MPtC inhibits platelet adhesion to LPS-stimulated endothelial cells in vitro and alleviates LPS-induced thrombosis in glomeruli in mice. Importantly, MPtC reduces mortality in LPS-challenged mice, suggesting a protective effect of this inhibitor during endotoxemia. Interestingly, MPtC, but not the integrin antagonist, Integrilin, alleviated LPS-induced thrombocytopenia.

Conclusions—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 antiplatelet strategy in managing endotoxemia. (Arterioscler Thromb Vasc Biol. 2013;33:2529-2537.)

Key Words: endotoxemia • GPIb-IX • lipopolysaccharide • platelet • platelet inhibitor • thrombocytopenia • thrombosis
Importance of GPIb-IX in LPS-Induced Mortality

We have used 2 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 GPIb-IX inhibition in the 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 interleukin-4 receptor (IL4R) extracellular domain and GPIbα transmembrane and cytoplasmic domain (IL4R-GPIbα). IL4R-GPIbα 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 GPIb−/− mice.25,28 Compared with wild-type mice, IL4R-GPIbα 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 (Figure 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-IX, we have developed micellar MPαC and its Effects on GPIb-IX–Dependent Platelet Function.

Materials and Methods

Materials and methods are detailed in the online-only Supplement. Myristoylated peptides MPαC (C13H27CONH-SIRYSGHpSL) and MCsC (C13H27CONH-LSSYSHGSR) were formulated as micelles with PEG2000/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 45:5:5:1 (in some experiments, 45:5:2) as previously described.24 The interleukin-4 receptor/βα mice lack the gene for endogenous mouse GPIbβ but instead express a fusion protein where the extracellular sequences of human GPIbβ, prevents LPS-induced glomerular microvascular thrombosis, 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.
similar pharmacokinetics in the circulation (Figure I in the online-only Data Supplement). 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 coimmunoprecipitation of GPIb-IX and 14-3-3ζ by ≈80% (Figure 2A and 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 pretreated 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 (Figure 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 adenosine diphosphate (ADP), collagen, and U46619 (a thromboxane A2 analog; Figure 2D). Thus, micellar MPαC selectively inhibits GPIb-IX–dependent platelet function without inhibiting general platelet activation signaling.

**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 (FeCl3)-induced mouse carotid artery thrombosis model. Retro-orbital injection of micellar MPαC significantly (P<0.01) delayed FeCl3-induced carotid artery occlusive thrombosis compared with the control (Figure 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 (Figure 3A–3C). However, LPS treatment of endothelial cells slowly and transiently induced platelet adhesion to HUVEC cells, with a maximal effect at 1 hour (Figure 3A). Treatment of platelets with micellar MPαC, but not the control micellar MCsC, inhibited LPS-induced platelet adhesion (Figure 3B and 3C). Similarly, an anti-GPIbα monoclonal antibody, LJ-P3, also inhibited platelet adhesion to LPS-stimulated HUVEC cells (Figure 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 (Figure 3E). These data suggest that LPS induces platelet adhesion to the vascular endothelium by leukocyte-dependent and leukocyte-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.

**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 C57BL 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 (Figure 4A). LPS-induced microvascular thrombosis was significantly reduced in micellar MPαC-treated mice, as compared with the MCsC micelles (Figure 4A). Similar results were also obtained with an immunohistochemical staining of microvascular thrombi with an antiplatelet integrin αⅡbβ3 antibody (Figure 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.

**Effect of Micellar MPαC and Integrin on Survival Rate in LPS-Challenged Mice**

The effects of MPαC in alleviating LPS-induced platelet–endothelial cell adhesion and microvascular thrombosis suggest 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 (Figure 5A). Injection of the control micellar MCsC had no significant effect on LPS-induced mortality (Figure 5A).
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In comparison, a single dosage of micellar MPαC injection before LPS challenge significantly reduced LPS-induced mortality to ≈30% (P=0.02; Figure 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 (Figure 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 (Figure 5C), indicating a role for the platelet-dependent microvascular thrombosis in the grave consequences of endotoxemia, and the beneficial effect of antiplatelet therapy. Although the effects of MPαC and Integrilin in reducing mortality are similar, injection of micellar MPαC to mice only mildly prolonged the tail bleeding time (Figure 5D). In contrast, Integrilin caused dramatically prolonged tail bleeding time in mice (Figure 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 toward bleeding side effect.

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 of LPS caused a platelet count decrease of ≈50% 4 hours after LPS injection (Figure 6A). A single injection of micellar MPαC, partially, but significantly mitigated LPS-induced thrombocytopenia (Figure 6A). In contrast to the significant effect of MPαC, injection of 5 mg/kg of Integrilin had no effect on LPS-induced thrombocytopenia (Figure 6B) despite its significant effect in prolonging bleeding time (Figure 5E) and in reducing mortality (Figure 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 (Figure 6C). These data indicate that LPS-induced thrombocytopenia involves GPIb-IX function but not that of integrin αIIBβ3. 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 patients with endotoxemia.

It is currently recognized that poor prognosis of endotoxemia in sepsis is associated with systemic microvascular thrombosis and disseminated intravascular coagulation. However, the mechanism that initiates microvascular thrombosis in endotoxemia remains unclear. The current concept
emphasizes the initiation of the coagulation cascade. Thus, the clinical antithrombotic treatment of severe sepsis is limited to anticoagulant 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 seem 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 antithrombotics 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 is that the animal...
endotoxia models may not precisely replicate the wide range of pathophysologic events occurring in humans. Thus, results obtained with mouse or other animal models, although very valuable as a guide and theoretical basis, need to be carefully evaluated in treating human endotoxia. 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. 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 ≈47% to 61% similarity in gene changes between human and mouse in response to inflammatory stimuli as shown in that study. 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 performing severe endotoxia models similar to the stringently controlled animal experiments is unethical in humans. Furthermore, it is well established that human, mouse, 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 endotoxia (and many other severe diseases) to obtain knowledge about the molecular mechanisms and discover drug targets for the treatment of human diseases. This approach has proven valuable. Abandoning of animal models by overemphasis of the difference between humans and animals will prove detrimental to our progress in understanding and treating severe human diseases.

To be able to further test whether GPIb-IX-mediated platelet adhesion and activation are important in severe endotoxia in humans, it is necessary to develop new drugs that can interfere with GPIb-IX function in vivo in patients with endotoxia. Incidentally, MP	auC 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, MP	auC acts on the cytoplasmic domain of GPIbα and does not induce thrombocytopenia. In fact, a major novelty of this study is the discovery that MP	auC alleviates thrombocytopenia induced by LPS. Micellar MP	auC represents not only a potential new strategy for the management of endotoxia-induced microvascular thrombosis and potentially for improving the survival rate of patients with sepsis 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. In inherited thrombotic thrombocytopenic purpura, VWF–GPIb interaction also causes microvascular thrombosis and thrombocytopenia. Thus, it would be interesting to further investigate whether MP	auC 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 MP	auC exerts its effects mainly through inhibiting 14-3-3 binding to GPIb, we do not totally exclude the possibility of additional effects of MP	auC on other functions of 14-3-3. However, such additional effects of MP	auC, if present, are also likely to involve the GPIb-IX signaling pathway because MP	auC does not significantly affect GPIb-IX–independent platelet activation and mimics the antithrombotic 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. It has been reported that platelets adhere to inflammatory mediator (histamine)-activated endothelial cells and form a beads-on-a-string–like structure attributable to exposure of ultralarge VWF multimers on the endothelial surface. 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 a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) has been reported in patients with sepsis. Also, increased VWF levels and the presence of more active ultralarge VWF in plasma have been found in severe sepsis and in patients with septic shock. In a sepsis model, VWF-deficient mice lived significantly longer than wild-type mice, 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 after LPS challenge. It is also possible that the role of GPIb-IX may be mediated by its binding to endothelial P-selectin and endothelial-bound coagulation factors (such as thrombin) or through leukocyte or endothelial integrins, although there have been studies challenging the role of P-selectin and integrins in platelet string formation on endothelial cells. It is interesting to further investigate the mechanism by which GPIb-IX mediates microvascular thrombosis in endotoxia and whether an antiplatelet therapy will have an advantage over anti-VWF therapy in treating platelet-dependent microvascular thrombosis.

Our data indicate not only that MP	auC inhibits LPS-induced microvascular thrombosis but also that it alleviates thrombocytopenia induced by LPS. Consumptive thrombocytopenia in sepsis and endotoxia is associated with poor prognosis. 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 the spleen. Interestingly, a recent study suggests that clearance of both VWF and platelets involves Ashwell receptors in hepatocytes. Thus, it is tempting to speculate
LPS-induced consumptive thrombocytopenia is partially dependent on 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 MPtC 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 be potentially effective in the management of endotoxemia in sepsis.

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Disclosures

X. D. holds a patent relevant to this study. The other authors report no conflicts.

References

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.
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Supplemental Fig. I. Plasma levels and clearance of micellar peptides following retro-orbital injection. C57BL/6 mice were anesthetized, retro-orbitally injected with micellar MPαC and MCsC (2 μl/Kg). Blood samples were collected at indicated time points. The concentration of MPαC and MCsC in mouse plasma was determined using high performance liquid chromatography and tandem mass spectrometry (LC/MS/MS).
Materials and Methods

Micellar peptide preparation. Myristoylated peptides MPαC (C13H27CONH-SIRYSGHpSL) and MCsC (C13H27CONH-LSISYGSHR) were synthesized in the Protein Research Laboratory, University of Illinois at Chicago. PEG$_{2000}$-DSPE (Avanti Polar Lipids Inc. Alabaster, AL), L-α-phosphatidylcholine (egg PC, Type XI-E, Sigma-Aldrich, St. Louis, MO) and peptides were mixed at a molar ratio of 45:5:1 (in some experiments, 45:5:2). Micelles were prepared using a film rehydration method as previously described.$^1$

Survival analysis in transgenic GPIbα mice. The generation and characterization of mouse colonies expressing GPIbα variants has been previously described$^{2,3}$. Briefly, the IL-4R/Ibα variant lacks the gene for endogenous mouse GPIbα but instead expresses a fusion protein where the extracellular sequences of human GPIbα have been replaced by an extracellular domain from the interleukin-4 receptor$^2$. The mice have a significant bleeding phenotype similar to the mouse model of the human Bernard-Soulier syndrome. LPS purified from E. coli (0111:B4, Product #62630, Sigma, St. Louis, MO) and dissolved in isotonic saline was injected intraperitoneally. The referenced LPS dosage, i.e., 100 µg, is based on the amount per 30 g of mouse bodyweight. Male mice of approximately 12 weeks of age were used. Kaplan Meier survival curves and log-rank analysis of the data are presented.

Platelet preparation. Human blood was drawn by venipuncture from healthy volunteers. Institutional Review Board approval was obtained from the University of Illinois at Chicago, and informed consent from volunteers was obtained in accordance with the Declaration of Helsinki. To prepare platelet-rich plasma (PRP), whole blood was anticoagulated with 3.8% trisodium
citrate. To prepare human platelets, one-seventh volume of ACD was used as anticoagulant. Platelets were washed twice and resuspended in modified Tyrode's buffer as previously described.

**Coimmunoprecipitation of GPIb-IX with 14-3-3ζ.** Micellar MPαC or control peptides (100 μM) were preincubated with washed human platelets (1X10⁹/mL) for 30 minutes. Platelets were then solubilized with a solubilization buffer, and lysates were immunoprecipitated with an anti-GPIb monoclonal antibody LJ-P3 (kindly provided by Dr Zaverio Ruggeri, The Scripps Research Institute) or IgG control. The immunoprecipitates were then immunoblotted with anti-GPIbα and anti-14-3-3ζ antibodies, respectively.

**Platelet adhesion to HUVECs under shear stress.** Human umbilical vein endothelial cell (HUVEC) monolayers were stimulated with 20 μM LPS (Sigma-Aldrich, St. Louis, MO) or vehicle control for various lengths of time. Washed platelets (3X10⁹/ml) were preincubated with micellar peptides (25 μM) or antibodies (10 μg/ml) for 15 minutes and then loaded onto HUVECs. In some experiments, platelet adhesion was analyzed in the presence of isolated human leukocytes (1X10⁹/ml). A cone-plate rheometer (Rheostress 1, Thermo-HAAKE, Paramus, NY) was used to introduce constant shear rate (800s⁻¹) to the platelets for 5 minutes. Dishes were then quickly rinsed with 5 ml PBS three times. Cells were fixed with 4% paraformaldehyde and visualized under a Leica DMI RB microscope using a 40X/0.55 NA objective. Adherent platelets were counted in 10 randomly selected fields. Statistical significance was analyzed using t-test.

**In vivo bleeding time, arterial thrombosis, and thrombocytopenia.** All animal studies are approved by the Institutional Animal Care Committee. C57BL/6 mice were anesthetized and retro-orbitally injected with micellar MPαC, scrambled peptide (2 μmol/kg) or Integulin.
(Integrilin, 5 mg/kg). After 15 minutes, tail bleeding time was determined using the previously described method. The bleeding time is defined as the time to stable cessation of bleeding with no re-bleeding within 1 minute. FeCl$_3$-induced carotid arterial thrombosis was studied as previously described. For thrombocytopenia, mice were treated with LPS (12 µg/g, I.P.). Approximately 1 mm tail end was snipped at 0, 2, and 4 hour time points and blood was collected. Platelet counts were analyzed by a Hemavet (HV950FS, Drew Scientific, Dallas, TX) multispecies hematology analyzer.

**Histologic analysis.** LPS (22 mg/kg) was administered intraperitoneally into C57BL/6 mice 15 minutes after retro-orbital injection of MPαC or MCsC (2 µmol/kg). After 24 hours, kidneys were perfused with phosphate-buffered saline and fixed in 10% formalin, detached, embedded in paraffin and sectioned at 5 µm. Slides were then stained with Mallory’s phosphotungstic acid hematoxylin method (PTAH) (Rowley Biochemical, Danvers, MA) to identify platelets and fibrin, and viewed with a Leica DMI RB microscope using a 40X/0.55 NA objective. Total thrombotic area/glomerulus was quantitated by analyzing 20 glomeruli from wild type mice and 50 glomeruli each from micellar MPαC and scrambled peptide treated mice (6 mice for each group). Platelets in thrombi were detected with a rat anti-mouse integrin α$_{IIb}$ monoclonal antibody (Santa Cruz, Santa Cruz, CA) using Vectastain ABC system and a peroxidase substrate DAB.

**Survival Assay.** C57BL/6 mice were retro-orbitally injected with MPαC or control peptide (2 µmol/kg) or Integrin (Integrilin, 5 mg/kg) 15 minutes prior to LPS (22 mg/kg) intraperitoneal injection and then monitored for 48 hours. In some experiments, C57BL/6 mice were first injected intraperitoneally with LPS (22mg/kg) 1 hour before the retro-orbitally injection of MPαC or control peptide (10µmol/kg). Survival rate (the percentage of living mice in the group at each time point) data were analyzed using Kaplan-Meier plot.


