Targeting Gα13-integrin interaction ameliorates systemic inflammation

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Systemic inflammation as manifested in sepsis is an excessive, life-threatening inflammatory response to severe bacterial or viral infection or extensive injury. It is also a thrombo-inflammatory condition associated with vascular leakage/hemorrhage and thrombosis that is not effectively treated by current anti-inflammatory or anti-thrombotic drugs. Here, we show that MB2mP6 peptide nanoparticles, targeting the Gα13-mediated integrin “outside-in” signaling in leukocytes and platelets, inhibited both inflammation and thrombosis without causing hemorrhage/vascular leakage. MB2mP6 improved mouse survival when infused immediately or hours after onset of severe sepsis. Furthermore, platelet Gα13 knockout inhibited septic thrombosis whereas leukocyte Gα13 knockout diminished septic inflammation, each moderately improving survival. Dual platelet/leukocyte Gα13 knockout inhibited septic thrombosis and inflammation, further improving survival similar to MB2mP6. These results demonstrate that inflammation and thrombosis independently contribute to poor outcomes and exacerbate each other in systemic inflammation, and reveal a concept of dual anti-inflammatory/anti-thrombotic therapy without exacerbating vascular leakage.

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Systemic inflammatory response syndrome can be triggered by a bacterial infection (such as sepsis) or by viral infection. Under these conditions, leukocytes and macrophages release large amounts of cytokines (cytokine storm), oxidants, and other pro-inflammatory factors, which not only cause systemic inflammatory responses and vascular leakage/hemorrhage, but also thrombosis. Inflammation, vascular leakage, and thrombosis together lead to multi-organ dysfunction including acute respiratory distress syndrome (ARDS) and kidney failure, disseminated intravascular coagulation (DIC), and circulatory system collapse resulting in morbidity and mortality. According to the Centers for Disease Control, one in three patients who die in a hospital have sepsis and at least 1.7 million adults develop sepsis annually in the U.S. which is fatal in ~270,000 cases. Similarly, viral infection can also be associated with systemic inflammation. Thus, there are urgent needs for new drugs to treat systemic inflammation. However, there is no effective drug treatment for systemic inflammation, including that coming from severe sepsis, despite intensive research and numerous clinical trials. Most of the failed efforts in anti-sepsis drug development have concentrated on anti-inflammatory/immune regulatory therapies, suggesting that there are possible inflammation-independent factors contributing to poor outcomes. Interestingly, the only novel drug approved (in 2001) for the treatment of severe sepsis is the recombinant activated protein C (APC, Xigris®), which inhibits thrombin generation, thrombosis, and certain aspects of inflammation. However, the clinical trials indicating improved outcomes in APC-treated septic patients also revealed a significant increase in adverse bleeding events. Post-market clinical studies further revealed significant adverse effect of bleeding in both adult and pediatric patients, outweighing the therapeutic effects. Subsequently, a repeat clinical trial showed no adverse effect of bleeding but also no efficacy, leading to its withdrawal from the market. These clinical trials suggest a potential benefit for antithrombotic drugs in sepsis treatment, which is consistent with the numerous data showing a close association between inflammation and thrombosis, and with the reported beneficial effects of anti-platelet drugs in treating sepsis in patients and animal models. These studies also suggest that the effective APC treatment is associated with adverse effect of hemorrhage, which counteracts the beneficial effect of this drug. Based on these basic and clinical studies, we hypothesize that inflammation, vascular leakage/hemorrhage, and thrombosis independently contribute to poor outcomes in systemic inflammation, in addition to inter-dependently exacerbating each other. This hypothesis also suggests that it would be more effective to target both inflammation and thrombosis without exacerbating vascular leakage/hemorrhage in treating systemic thrombo-inflammatory conditions.

The integrin family of cell adhesion receptors plays important roles in the functions of platelets and leukocytes. We have shown previously that the integrin α13β2, directly interacts with an ExE motif in the cytoplasmic domains of integrin β2 subunits and this binding is selectively important in stimulating integrin outside-in signaling, without affecting inside-out signaling and the ligand-binding function of integrin α13β2. Integrin α13β2 is important in thrombosis but apparently dispensable for hemostasis, and peptides derived from the β2 ExE motif potentially inhibited thrombosis without affecting hemostasis in mouse models. A form of the ExE motif is also present in leukocyte β2 integrins, which are critically important in inflammation.

In this work, we explore the effect of a peptide (MB2mP6) derived from the β2 ExE motif and the effects of leukocyte and platelet α13 knockout on systemic thrombo-inflammatory conditions using a mouse sepsis model induced by cecal ligation-puncture (CLP). Our data demonstrate that MB2mP6 inhibits α13β2-integrin interaction and integrin outside-in signaling of both β2 integrins in leukocytes and β3 integrins in platelets without affecting inside-out signaling and adhesion function of leukocytes and platelets. Importantly, MB2mP6 inhibits both microvascular thrombosis and inflammatory cytokine release and improves survival rates in the CLP-induced sepsis model in mice. Furthermore, platelet selective knockout of α13 integrates thrombosis with only moderate effect on inflammation, whereas leukocyte-selective knockout of α13 integrates inflammation with minimal effect on thrombosis. Leukocyte- or platelet- selective knockout of α13 only moderately increases the survival rate of septic mice. In contrast, dual leukocyte and platelet knockout of α13 integrates both thrombosis and inflammation and markedly improves the survival rate of mice in CLP sepsis, similar to that found with MB2mP6. These results support the hypothesis that thrombosis and inflammation are independently and inter-dependently important in the outcome of the systemic thrombo-inflammatory conditions and introduce a concept of dual anti-inflammatory/anti-thrombotic therapy that does not exacerbate vascular leakage for treating systemic inflammation.

Results

MB2mP6 inhibits LPS-induced macrophage cytokine expression in vitro. Integrins play critical roles in platelet and leukocyte functions. We previously discovered that ligand binding to integrin α13β2 induces binding of a G-protein subunit, Gα13, to β2, transmitting outside-in signaling. Because outside-in signaling is a post-adhesion amplification mechanism, inhibiting this process minimally affects primary integrin-mediated platelet aggregation and hemostasis. Gα13 binds to a cytoplasmic ExE motif which is homologous among β2 and β3 integrins. We thus designed an inhibitory peptide, MB2mP6 (Myr-KEKEKL), based on the ExE sequence of integrin β2. MB2mP6 was formulated into high-loading peptide nanoparticles (HLPN) for efficient intracellular delivery in vitro and in vivo. MB2mP6 inhibited Gα13 co-immunoprecipitation with β2 integrins in LPS-stimulated macrophages differentiated from the human monocytic leukaemia cell line THP-1 cells. Strikingly, MB2mP6 HLPN potently inhibited LPS-induced expression of proinflammatory cytokines IL-1β, IL-6, IL-12 p40, and TNFα in THP-1 differentiated macrophages (Fig. 1b-e). MB2mP6 also inhibited LPS-stimulated cytokine IL-1β and IL-6 expression in mouse bone marrow-derived macrophages (BMDM; Supplementary Fig. 1a, b), suggesting that Gα13-integrin interaction is important in macrophage proinflammatory function. To investigate whether the inhibitory effect of MB2mP6 on cytokines is related to cell adhesion, which is known to be integrin-dependent, we compared the cytokine expression induced by LPS under conditions of adhesion and suspension. The expression of inflammatory cytokines IL-1β, IL-6, IL-12 p40, and TNFα induced by LPS was significantly lower in suspended THP-derived macrophages than in adherent cells (Fig. 1f–i), suggesting that cell adhesion greatly promotes cytokine expression. We further investigated whether MB2mP6 affects macrophage adhesion. THP-1-differentiated macrophages were pretreated with MB2mP6 HLPN or scrambled peptide control for 20 min and then loaded onto 48-well plates pre-coated with integrin ligand human (h)ICAM-1 (10 μg mL⁻¹). MB2mP6 had no effect on the adhesion of THP-1 differentiated macrophages on hICAM-1 (Fig. 1j). These data suggest that MB2MP6 inhibits post-adhesion signaling events, which is outside-in signaling, and thus macrophage cytokine expression. Leukocyte adhesion is important in phagocytosis.
Fig. 1 MB2mP6 blocks $\alpha_{13}$ interactions with $\beta_2$ integrins and inhibit adhesion-dependent macrophage cytokine expression. a Representative immunoblots (from three independent experiments) of anti-$\beta_2$ antibody-co-immunoprecipitated integrin $\beta_2$ subunit and $\alpha_{13}$ in macrophages differentiated from PMA-stimulated THP-1 cells. The macrophages were pretreated with MB2mP6 or control peptide HLPN (50 $\mu$M) for 20 min and then stimulated with LPS (100 ng mL$^{-1}$) for the indicated time prior to immunoprecipitation. Immunoprecipitates and total cell lysates were then immunoblotted with both anti-$\beta_2$-integrin and anti-$\alpha_{13}$ antibodies. b–e Comparison between MB2mP6 HLPN and scrambled peptide control (Scra) in Il-1$\beta$ (b), Il-6 (c), Il-12 p40 (d), and Tnf$\alpha$ (e) mRNA expression in THP-1-derived macrophages stimulated with 100 ng mL$^{-1}$ LPS. The mRNA expression was detected by qRT-PCR (all groups, $n=4$, independent cultures). f–i Comparison between adherent and non-adherent THP-1-derived macrophages in the levels of cytokine Il-1$\beta$ (f), Il-6 (g), Il-12 p40 (h), and Tnf$\alpha$ (i) expression as detected by qRT-PCR (all groups, $n=3$, independent cultures). j No effect of MB2mP6 pretreatment (20–100 $\mu$M) on adhesion of THP-1-derived macrophages onto hICAM-1 (10 $\mu$g mL$^{-1}$) precoated in 48-well plates (all groups, $n=4$, independent cultures). All data are shown as mean ± SEM. Data in (b–j) were analyzed by two-way ANOVA with the post hoc Bonferroni’s multiple comparisons test (b–e) or Sidak’s multiple comparisons (f–i) or Tukey’s multiple comparisons tests (j).
MB2mP6 inhibits integrin outside-in signaling in platelets. To test whether MB2mP6 also inhibits Gα13-dependent outside-in signaling of the platelet integrin αIIbβ3, Gα13 was co-immunoprecipitated with β3 integrins in thrombin-stimulated human platelets that had been preincubated with MB2mP6 or control peptide. MB2mP6 inhibited Gα13-β3 integrin interaction (Fig. 3a). MB2mP6 also dose-dependently inhibited human platelet granule secretion and secretion-dependent secondary aggregation induced by low-dose thrombin (Fig. 3b, c). However, MB2mP6 had no effect on platelet aggregation (Fig. 3d) or ATP secretion (Fig. 3e) induced by high concentrations of thrombin nor on fibrinogen binding to platelets induced by PAR4-agonist peptide (Fig. 3f, g). MB2mP6 also did not affect ADP-induced platelet aggregation (Fig. 3h), which is minimally dependent on outside-in signaling in the presence of physiological calcium concentration. These data suggest that MB2mP6 does not directly affect outside-out signaling nor the ligand binding to integrin αIIbβ3, which mediates primary aggregation, but selectively inhibits the secondary amplification of platelet aggregation, which is mediated by outside-in signaling. Interestingly, whereas MB2mP6 HLPN did not significantly affect ATP secretion induced by a high dose of thrombin (Fig. 3e), the platelet surface P-selectin expression was still inhibited by MB2mP6 at both low and high dose thrombin stimulation (Fig. 3i).

MB2mP6 inhibits thrombosis but does not cause bleeding in vivo. We used the ferric chloride-induced mouse carotid artery thrombosis model to determine the in vivo effects of MB2mP6 in inhibiting thrombosis. MB2mP6 effectively inhibited FeCl3-induced mouse carotid artery thrombosis in vivo, although the anti-thrombotic effect appeared to be less potent than the β3-derived M3mP6 peptide (Fig. 4a). Importantly, MB2mP6 neither affected injury-induced tail-bleeding time (Fig. 4b), nor inflammation-induced hemorrhage in the reverse passive Arthus (rpA) reaction in mice (Fig. 4c). These data demonstrate that MB2mP6 is anti-inflammatory and anti-thrombotic but does not cause or exacerbate hemorrhage.

MB2mP6 enhances survival in a CLP severe sepsis model. We used the mouse cecal ligation and puncture (CLP) model of severe polymicrobial sepsis to test the possible therapeutic effects of MB2mP6 on systemic inflammation. MB2mP6 or a scrambled control peptide HLPN were infused i.v. immediately after CLP at a rate of 1.25 µmol kg⁻¹ h⁻¹ through a pre-placed jugular vein cannula (Fig. 5a). To mimic clinical treatment, antibiotic therapy (Claforan solution) was also started at the same time with both groups. The MB2mP6 group showed a significant improvement in survival probability (survival rate 71% in the MB2mP6 group vs 26% in the control peptide group at 192 h (8 days); p = 0.002) (Fig. 5a). The control peptide group was similar to the saline treatment group (Fig. 5a). To more closely mimic the clinical conditions in sepsis treatment, we started MB2mP6 infusion 6 or 18 h after CLP onset. When started at 6 h after CLP, MB2mP6 also significantly improved the 8-day survival probability (survival rate 42% in the MB2mP6 group vs 6.25% in the control group, p = 0.003, Fig. 5b). Even when started at a very late 18 h after CLP onset, MB2mP6 treatment still resulted in significant improvement in survival probability compared to a scrambled peptide control group (survival rate 6.7% in the MB2mP6 group vs 0% in the control group, p = 0.011, Fig. 5c), although the percentages of survival further decreased in both MB2mP6 and control groups. The decrease in survival rate at 18 h was likely caused by the delayed start of antibiotic treatment, which was in the treatment regimen together with MB2mP6 or control peptide HLPN. In testing the inflammation and coagulation states of the septic mice at the time of treatment after starting CLP, we found that the proinflammatory cytokines, IL-6, and even the late phase cytokine TNFα, significantly increased at 6 h (Supplementary Fig. 2a, b). At 18 h, IL-6 had already decreased to close to base line level, whereas TNFα further increased. The “anti-inflammatory” cytokine IL-10 was near the baseline at 6 h but significantly increased at 18 h suggesting entry into the severe “suppression” phase at this late time point (Supplementary Fig. 2c). Fibrinogen and thrombin-anti-thrombin complex (TAT) had already increased at 6 h and further increased at 18 h, suggesting “hypercoagulant” state (Supplementary Fig. 2d, e). The fibrinolytic product D-dimer only became elevated at 18 h (Supplementary Fig. 2f), consistent with its association with a more grave stage of sepsis. These results suggest that systemic inflammation had fully developed at 6 h after sepsis onset, and reached the late severe stage associated with DIC at 18 h. Even at these late phases, MB2mP6 treatment still had a beneficial effect. Thus, MB2mP6 effectively treats and improves the survival probability of severely septic mice.
MB2mP6 inhibits inflammation and thrombosis during sepsis.
To assess the effect of MB2mP6 on inflammation during sepsis in vivo, we tested pro-inflammatory cytokine levels in mouse serum collected 24 h after CLP. Control mice showed significantly increased secretion of IL-6 and TNF-α, which were significantly reduced by MB2mP6 treatment (Fig. 5d, e). Similarly, expression of IL-6 and TNF-α transcripts in septic mouse lung at 24 h were also induced by CLP and significantly suppressed by MB2mP6.
infusion (Supplementary Fig. 3a, b). These data demonstrate that MB2mP6 has potent anti-inflammatory effects in vivo during severe sepsis.

Severe sepsis causes microvascular thrombosis in kidney glomeruli, damaging kidney function. Indeed, in CLP septic mice, we observed microvascular thrombosis in kidney glomeruli, as indicated by Mallory’s phosphotungstic acid hematoxylin (PTAH) staining (Fig. 5f), anti-fibrin staining of fibrin deposition, anti-αHb staining of platelets, and anti-VWF staining (Supplementary Fig. 4a–c). CLP sepsis also impaired renal function as indicated by elevation of BUN (Fig. 5g), creatinine, and cystatin C (Supplementary Fig. 4d, e) in the control group 24 h after CLP. MB2mP6 treatment significantly inhibited glomerular thrombosis and reduced BUN, creatinine, and cystatin C (Fig. 5f, g and Supplementary Fig. 4a–c). MB2mP6 also decreased the elevated TAT levels without affecting fibrinogen concentrations in the whole blood of septic mice 24 h after treatment (Supplementary Fig. 5a, b). Thus, MB2mP6 inhibits both inflammation and thrombosis and improves renal function in septic mice.

**Fig. 4** The effects of MB2mP6 on FeCl₃-induced carotid artery thrombosis and on hemorrhage in vivo. a. b Comparison of the effects of MB2mP6 and M3mP6 (Mtr-FFERL) on FeCl₃-induced carotid artery thrombosis (a) and tail-bleeding time (b) (control, n = 29; MB2mP6, n = 14; M3mP6, n = 29, independent animals). c Comparison between MB2mP6 and scrambled peptide on inflammation-induced hemorrhage in the rpA assay (n = 9 for all groups, independent animals). All data are shown as mean ± SEM. Data in (a), (b) and (c) were analyzed by unpaired Mann–Whitney test (two-tailed).

**MB2mP6 reduces septic lung vascular leak and does not cause bleeding.** An important cause of mortality in sepsis and other systemic and pulmonary inflammatory conditions is the ARDS, in which inflammation-induced vascular leakage (hyperpermeability) and hemorrhage acutely cause severe lung edema, impairing respiratory function. To test whether MB2mP6 protected from lung vascular leak in septic mice, we examined the lung permeability 24 h after CLP. MB2mP6 treatment significantly reduced Evan’s blue infiltration into the lung as compared with control peptide when treatment started immediately after CLP (Fig. 6a) and when treatment started 6 h after CLP (Fig. 6b). Thus, MB2mP6 alleviates the vascular leak in septic lungs. To investigate the effects of MB2mP6 on bleeding during CLP-induced sepsis, we measured the hemoglobin (Hb) contents in the stools collected from septic mice 24 h after CLP onset. Indeed, hemoglobin levels in the stools of septic mice were elevated 24 h after CLP surgery compared to before surgery, and the Hb level was not affected by MB2mP6 treatment as compared with scrambled peptide control (Supplementary Fig. 6). Taken together, these data suggest that MB2mP6 attenuates vascular hyperpermeability/vascular leakage during severe sepsis with no adverse effect on bleeding. These results indicate that MB2mP6 may be potentially effective in preventing ARDS in systemic inflammation.

**Effects of selective α₁₃ knockout in leukocytes or platelets on sepsis.** The beneficial effects of MB2mP6 in treating sepsis suggest that Gα₁₃-integrin interaction in either leukocytes or platelets or both are important in the development of systemic inflammation. To test the importance of leukocyte Gα₁₃, we generated leukocyte-specific Gα₁₃ knockout mice by mating Gα₁₃−/− mice generously provided by Dr. Stefan Offermanns, Max Planck Institute for Heart and Lung Research, Germany, with LysM-Cre mice. The western blotting analysis confirmed the absence of Gα₁₃ protein in both macrophages and neutrophils but not platelets of Gα₁₃−/−LysMcCre mice (Supplementary Fig. 7a, b). Leukocyte-specific Gα₁₃ knockout (Gα₁₃−/−LysMcCre) mice had a moderately but significantly improved 8-day survival rate in CLP-induced sepsis as compared with control groups (44% in Gα₁₃+/+ control group, p = 0.044 between Gα₁₃+/+ and Gα₁₃−/−LysMcCre) mice vs 20% in the LysMcCre only group vs 19% in the Gα₁₃−/−LysMcCre group (Fig. 7a) and the elevation of the kidney damage marker BUN was also not reduced in the blood of Gα₁₃−/−LysMcCre septic mice (Fig. 7c). These data suggest that leukocyte Gα₁₃ is not a critical factor in kidney microvascular thrombosis and damage and that the protective effect of leukocyte-specific Gα₁₃ knockout on septic systemic inflammation was mainly due to inhibition of leukocyte-mediated cytokine secretion and inflammation.
To assess the contribution of platelet Gα13 to the therapeutic effect of MB2mP6, and its role in systemic inflammation, platelet-specific knockout mice were generated by mating Gα13fl/fl mice with PF4-Cre mice (Supplementary Fig. 7a, b). The 8-day (192 h) survival rate in the platelet-specific Gα13 knockout (Gα13fl/fl-PF4Cre) mice after CLP sepsis was moderately but significantly improved compared with control PF4-Cre or Gα13fl/fl mice (39.2% vs 7.6% in the PF4-Cre control group; \( p = 0.001 \)) (Fig. 7f), but significantly lower than MB2mP6-treated mice (Fig. 5a vs Fig. 7f and summarized in Supplementary Fig. 8, \( p < 0.05 \)). In contrast to leukocyte-specific Gα13 knockout, the CLP-induced glomerular microvascular thrombosis and kidney function impairment (indicated by blood BUN levels 24 h after CLP) were both markedly reduced in Gα13fl/fl-PF4Cre mice compared with Gα13fl/fl mice (Fig. 7g, h). Thus platelet Gα13 plays an important role in glomerular microvascular thrombosis and kidney injury during sepsis, and is likely responsible for the therapeutic effect of MB2mP6 on glomerular thrombosis and kidney injury. However, the serum level of the inflammatory cytokine TNFα was not significantly reduced in Gα13fl/fl-PF4Cre mice (Fig. 7i), although cytokine IL-6, levels in mouse serum were partially (and significantly) reduced (Fig. 7j). Thus, it appears that platelet-
Fig. 5 MB2mP6 improves survival in a CLP sepsis model and reduces inflammatory cytokines, thrombosis and prevents sepsis-induced organ injury. 

**a** Effect of MB2mP6 and scrambled peptide (Scra) treatment immediately after CLP surgery on survival of C57BL/6 septic mice (Sham, n = 6; Scra, n = 23; MB2mP6, n = 24, independent animals). Log-rank (Mantel-Cox) test using GraphPad Prism software (two-tailed). **b** Effect of MB2mP6 and scrambled peptide (Scra) treatment 6 h after CLP onset on survival of C57BL/6 septic mice (sham, n = 6; Scra, n = 16; MB2mP6, n = 19, independent animals). Log-rank (Mantel-Cox) test using GraphPad Prism software (two-tailed). **c** Effect of MB2mP6 and scrambled peptide (Scra) treatment 18 h after CLP onset on survival of C57BL/6 septic mice (Sham, n = 6; Scra, n = 12; MB2mP6, n = 15, independent animals). Log-rank (Mantel-Cox) test using GraphPad Prism software (two-tailed). In (a), (b), and (c), “Sham” indicates mice receiving sham surgery (without CLP). **d** MB2mP6 inhibits protein levels of cytokines IL-6 and TNFα in mouse serum measured by ELISA 24 h after CLP (sham, n = 6; Scra, n = 8; MB2mP6, n = 10, independent animals). **e** Representative images of fibrin deposition detected by PTAH staining in mouse kidney glomeruli 24 h after CLP. Bars indicate the percentage of PTAH positive stained area per glomerulus (30 random glomeruli from 6 mice/group). Scale bar = 100 μm. **f** Plasma level of BUN in septic mice 24 h after CLP: comparison between MB2mP6-treated mice and scrambled peptide-treated mice (sham, n = 6; Scra, n = 9; MB2mP6, n = 9, independent animals). All data are shown as mean ± SEM. Data in (d) were analyzed by student t-test (two-tailed); data in (e) and (f) were analyzed by one-way ANOVA with post hoc Tukey’s multiple comparisons test.

Fig. 6 MB2mP6 inhibits CLP-induced lung vascular leakage induced by CLP sepsis. 

**a** Representative images of septic mouse lungs 24 h after CLP. The mice were treated with scrambled peptide (Scra) or MB2mP6 immediately after CLP onset. At 23.5 h after CLP, the mice were i.v. injected with Evans blue albumin (EBA; 1%, 25 mg kg−1 body weight). After 30 min, the mouse lungs were harvested, and the Evans blue was extracted by incubating with formamide at 65 °C for 18 h. The EBA contents in the lung tissues were calculated using a standard EBA curve detected at 620 nm absorbance with 740 nm reference (sham, n = 4; control, n = 5; MB2mP6, n = 4, independent animals). **b** Representative images of septic mouse lungs 24 h after CLP treated with Scrambled peptide or MB2mP6 6 h after CLP onset. The Evans blue contents in septic mouse lung 24 h after CLP were analyzed as in (a) (sham, n = 4; control, n = 6; MB2mP6, n = 6, independent animals). All data are shown as mean ± SEM. Data were analyzed by one-way ANOVA with Tukey’s multiple comparisons test.

**specific Ga13 knockout moderately reduced mortality in septic mice mainly by inhibiting microvascular thrombosis, although platelet Ga13 may also contribute to the exacerbation of the inflammatory state.**

**The effect of dual leukocyte and platelet Ga13 knockout on sepsis.** To further determine whether Ga13 in platelets and in leukocytes has independent roles in sepsis-induced mortality and organ damage, we generated platelet/leukocyte dual Ga13 knockout mice (Ga13−/− LysM−/−PF4−/− double Cre mice, Supplementary Fig. 7a, b). The 8-day survival rate after CLP in the dual Ga13 knockout mice was 66% (Fig. 8a, p = 0.0072 vs 29.4% for LysM−/−PF4−/− double Cre mice; p = 0.0001 vs 5.9% for Ga13−/− mice), similar to that of septic wild type mice treated with MB2mP6 (71%; Fig. 5a). This was also significantly higher than not only the control mice but also platelet-specific Ga13 knockout (39.2%) (Fig. 7f) vs Fig. 8a and summarized in Supplementary Fig. 8, p < 0.05) and leukocyte-specific Ga13 knockout mice (44%) (Fig. 7a vs Fig. 8a and summarized in Supplementary Fig. 8, p < 0.05). Consistent with these results, the elevation of kidney injury marker BUN (Fig. 8b) and cytokines IL-6 (Fig. 8c) or TNFα (Fig. 8d) in the blood of control mice 24 h after CLP were markedly inhibited in the dual Ga13 knockout mice. The CLP-induced glomerular...
**Fig. 7 Leukocyte- or platelet-specific \( \alpha_{13} \) knockout reduces mortality in CLP-induced septic mice.**

**a** The survival rate of mice after CLP in \( \alpha_{13}^{fl/fl} \)-LysMCre (leukocyte-specific \( \alpha_{13} \) knockout) mice compared to LysM-Cre or \( \alpha_{13}^{fl/fl} \) control mice. LysM-Cre sham, \( n = 6 \); LysM-Cre CLP, \( n = 22 \); \( \alpha_{13}^{fl/fl} \)-LysMCre sham, \( n = 6 \); \( \alpha_{13}^{fl/fl} \)-LysMCre CLP, \( n = 25 \). Log-rank (Mantel-Cox) test (two-tailed).

**b**, **c** Serum levels of IL-6 and TNF\( \alpha \) in septic mice 24 h after CLP in \( \alpha_{13}^{fl/fl} \)-LysMCre and \( \alpha_{13}^{fl/fl} \) control mice. All sham groups, \( n = 6 \); all CLP groups, \( n = 10 \) (independent animals).

**d** Images and quantification of immunohistochemical staining of fibrin deposition in mouse kidney 24 h after CLP. Bar graph shows the percentage of fibrin-stained area per glomerulus (30 random glomeruli from 6 mice/group, all groups, \( n = 30 \), independent glomeruli). Scale bar = 100 \( \mu \)m.

**e**, **f** Plasma level of BUN in septic mice 24 h after CLP in \( \alpha_{13}^{fl/fl} \)-PF4Cre mice and \( \alpha_{13}^{fl/fl} \) mice (all sham groups, \( n = 5 \); all CLP groups, \( n = 10 \), independent animals).

**g** Survival rate in mice after CLP-induced sepsis in \( \alpha_{13}^{fl/fl} \)-PF4Cre (platelet-specific knockout) mice compared to PF4-Cre or \( \alpha_{13}^{fl/fl} \) control mice. PF4-Cre sham, \( n = 6 \); PF4-Cre CLP, \( n = 25 \); \( \alpha_{13}^{fl/fl} \)-PF4Cre sham, \( n = 7 \); \( \alpha_{13}^{fl/fl} \)-PF4Cre CLP, \( n = 17 \). Log-rank (Mantel-Cox) test (two-tailed).

**h**, **i** Plasma levels of BUN in septic mice 24 h after CLP in \( \alpha_{13}^{fl/fl} \)-PF4Cre mice compared to \( \alpha_{13}^{fl/fl} \) mice (all groups, \( n = 9 \), independent animals).

**j** Serum levels of TNF\( \alpha \) and IL-6 in septic mice 24 h after CLP in \( \alpha_{13}^{fl/fl} \)-PF4Cre and \( \alpha_{13}^{fl/fl} \) control mice (all groups, \( n = 8 \), independent animals). All data are shown as mean ± SEM. Data in (b), (c), (h-j) were analyzed by two-way ANOVA with Tukey’s multiple comparisons test; data in (d) and (g) were analyzed by a two-tailed Mann-Whitney test.
Fig. 8 Platelet and Leukocyte dual-specific Gα13 knockout reduces inflammatory cytokines, thrombosis, and renal injury and improves survival in a mouse CLP sepsis model. a Survival rate in mice after CLP-induced sepsis in Gα13fl/−/PF4/LysMCre mice compared to LysM & PF4-double Cre or Gα13fl/fl control miceDouble Cre sham, n = 6; double Cre CLP, n = 16; Gα13fl/− CLP, n = 17; Gα13fl/−/PF4/LysMCre sham, n = 7; Gα13fl/−/LysMCre CLP, n = 19 (all groups, independent animals). Log-rank (Mantel-Cox) test (two-tailed). b Plasma levels of BUN in septic mice 24 h after CLP in Gα13fl/−/PF4/LysMCre mice compared to Gα13fl/fl mice. All groups, n = 9. c, d Serum levels of IL-6 and TNFα in septic mice 24 h after CLP in Gα13fl/−/PF4/LysMCre mice compared with Gα13fl/fl control mice. All groups, n = 9. e Images and quantification of immunohistochemical staining of fibrin deposition in mouse kidney 24 h after CLP. Bar graph shows the percentage of fibrin-positive stained area per glomerulus (30 random glomeruli from 6 mice/group, all groups, n = 30, independent glomeruli). Scale bar = 100 µm. All data are shown as mean ± SEM. Data in (b–d) were analyzed by two-way ANOVA with Tukey's multiple comparisons test; data in (e) were analyzed by a two-tailed Mann-Whitney test.

microvascular thrombosis was also significantly reduced in the dual Gα13 knockout mice as indicated by greatly reduced fibrin deposition (Fig. 8e). These data not only further support the distinct roles of Gα13 in platelets and leukocytes in septic systemic inflammation, but also demonstrate that their roles in sepsis are additive. Furthermore, these data demonstrate that the effectiveness of MB2mP6 in treating septic systemic inflammation in the CLP model is likely due to its ability to inhibit Gα13-integrin interaction and outside-in signaling in both platelets and leukocytes.

Discussion

In this study, we demonstrate that Gα13 and its interaction with leukocyte and platelet integrins play important roles in the thrombotic and inflammatory functions of platelets and leukocytes respectively during systemic inflammation. The leukocyte-mediated inflammatory response and platelet-dependent thrombosis both independently contribute to the poor outcome in systemic inflammation in addition to their interdependent roles in exacerbating each other as shown using the CLP model of sepsis in mice. We further show that a β2 integrin-derived peptide, MB2mP6, by targeting and inhibiting the Gα13 interaction with β2 and β3 integrins in leukocytes and platelets, effectively inhibits both thrombosis and inflammation to increase the survival probability of severely septic mice. The protective effect of MB2mP6 was shown even when the treatment was started in a late phase of sepsis. Importantly, MB2mP6 does not adversely affect hemostasis and even reduces pulmonary vascular leakage, a major mechanism leading to ARDS. To our surprise, MB2mP6, while inhibiting integrin-dependent cytokine secretion, did not significantly affect the phagocytosis/killing of bacteria by leukocytes, which likely contributes to its beneficial effect on sepsis. Thus our study provides strong support for a concept of simultaneously inhibiting inflammation and thrombosis while reducing vascular leakage and maintaining hemostatic function as an effective strategy for treating systemic inflammation.

Classically, Gα13 is coupled to heptahedral G protein-coupled receptors to mediate activation of the RhoA signaling pathway and contraction31–33. More recently, we discovered that Gα13 also directly interacts with homologous ExE motifs in the cytoplasmic domains of several integrin β subunits including platelet αIIbβ3, and β3 in leukocytes23,24. Gα13 binding to β3 in platelets does not affect inside-out signaling which is important for the activation of ligand binding to integrin αIIbβ3, nor the ligand-binding function of integrin αIIbβ3, but selectively inhibits the ligand-binding-induced outside-in signaling23. Mutations in the ExE motif abolishing the Gα13–β3 interaction selectively inhibited secondary amplification of platelet aggregation and cell spreading without affecting primary platelet adhesion and aggregation, which are required for primary hemostasis23. Thus, the β3-derived ExE motif peptides potently inhibited thrombosis without affecting normal hemostasis, unlike all current anti-platelet drugs23,25. Here we further show that the β2-derived ExE motif peptide MB2mP6 inhibits Gα13 binding to β3 integrins and potently inhibits macrophage cytokine synthesis. We also show that that cytokine expression in macrophage-like cell lines is adhesion-dependent, but MB2mP6 had no effect on adhesion per se, suggesting its effect only on outside-in signaling. Both MB2mP6 and
Gα13 knockout in leukocytes greatly reduced cytokine levels in CLP-induced septic mice. Thus, Gα13 is important in stimulating the cytokine storm during systemic inflammation. Importantly, MB2mP6 inhibited Gα13-integrin interaction both in platelets and leukocytes, and inhibited both inflammation and thrombosis without affecting hemostasis. Thus, MB2mP6 has the potential to be developed into a dual anti-thrombosis/anti-inflammatory drug that does not cause vascular leakage/bleeding.

It has been recognized that severe infection or extensive injury may induce excessive inflammatory responses intertwined with vascular leakage, hemorrhage, and thrombosis (vasculopathy), which stimulate and exacerbate each other in a vicious cycle developing into a systemic thrombo-inflammatory condition with high mortality. Once this systemic response has developed, however, anti-inflammatory drugs have been proven ineffective in improving the final outcome. Our results showing independent and additive effects of leukocyte-mediated inflammation and platelet-dependent thrombosis in severe sepsis provide an explanation for past failures of anti-inflammatory drugs in treating sepsis, and importantly provide strong support for an approach of simultaneously anti-inflammatory and anti-thrombotic therapy. However, an important feature of systemic inflammation is vascular leakage and hemorrhage, which are greatly exacerbated by all current anti-thrombotic drugs and have been shown to outweigh the beneficial effect of Xigris in the previous studies. In contrast, we demonstrate that MB2mP6, targeting the outside-in signaling of integrins both in leukocytes and platelets, does not exacerbate hemorrhage, but effectively reduces the vascular leakage in the lung. Importantly, we demonstrate that MB2mP6 is effective in treating septic systemic inflammation in the CLP mouse model, reducing both inflammation and thrombosis to enhance survival. These conceptual and translational advances thus set the stage for further studies to determine whether this new drug is effective in treating systemic inflammation in humans.

It is important to note that MB2mP6’s effectiveness is not limited to preventing sepsis immediately after CLP onset, but also has significant therapeutic effects when administered 6 and even 18 h after CLP in mice. In mice, sepsis-induced increases in most cytokines as well as lung neutrophil recruitment, alveolar leak, endothelial damage, liver neutrophil and platelet recruitment with impaired sinusoidal perfusion, and acute kidney injury are all well established by 4–6 h post-CLP. Our data showing elevation of pro-inflammatory cytokines, IL-6 and TNFα as well as the hypercoagulant state at 6 h and elevation of late anti-inflammatory cytokine IL-10 and D-dimer at 18 h, are consistent with these reports. Thus, MB2mP6 is not purely preventative, but rather disrupts the progression of systemic inflammation. However, anti-inflammatory/anti-thrombotic therapy will likely be successful if used before the grave “suppression” phase of the disease. If the patients/experimental animals have succumbed to the consequences of inflammation and thrombosis (e.g., multiple organ failure), they are unlikely to be revived with these (or likely any other) drugs. Indeed, the start of the treatment with MB2mP6/antibiotics immediately after sepsis onset resulted in better survival compared to infusion 6 or 18 h after CLP. Thus, the use of this type of drug early is likely to be more effective in disrupting the progression of systemic inflammation into the irreversible phase.

Overall, our data reveal the independent and interdependent roles of inflammation, vascular leakage/hemorrhage, and thrombosis in systemic inflammation and provides a concept and experimental drug for simultaneous anti-inflammatory and anti-thrombotic therapy without causing vascular leakage and hemorrhage. This therapeutic approach should help our defense against systemic inflammation, which is increasingly a challenge to human health.

**Methods**

**Animals.** Mice used in this study were 8- to 16-weeks-old with an equal sex ratio. C57BL/6 mice were gifts obtained from Dr. Stefan Offermanns’ lab (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany). PF4-Cre mice, LysM-Cre (Ly22-Cre), and C57BL/6 mice were obtained from the Jackson Laboratory. Platelet-specific or leukocyte-specific Gα13 knockout mice were generated by breeding Gα13fl/fl and PF4-Cre or LysM-Cre mice and confirmed by genotyping. Peripheral blood and western blot analysis (Supplementary Fig. 7) were Cre only without the Gα13, floxed allele or negative for Cre recombinase with matched genetic background, age, and sex. Animals were housed and bred in the Biologic Resources Laboratory at the University of Illinois at Chicago under 12 h light-dark cycles, controlled temperature (~23 degrees) and 40–50% humidity with free access to food and water. All animal procedures complied with the animal care and usage standards set forth by the National Institutes of Health and were approved by the Institutional Animal Care Committee, University of Illinois at Chicago (animal protocol number: 19-215). A randomized approach of choosing mice was used throughout the study, using all mice with the correct genotype without bias.

**Reagents.** Mouse anti-integrin β3 antibody (1.BB.246, sc-71397, 2 μg per 500 μg lysate) and rat anti-integrin αIIb antibody (mWReg30, sc-19963, 1:250 for IHC staining) were purchased from Santa Cruz Biotechnology. Rabbit anti-integrin β3 monoclonal antibody (D4N52, #73663, 1:1000 for western blot) was purchased from Cell Signaling Technology. Mouse anti-integrin β3 monoclonal antibody M15 against αIIb (Dr. Mark Davis) and rabbit anti-integrin β3 antibody (18309-1-AP, 1:1000 for western blot) was obtained from Protextech. Rabbit anti-Ga13 antibody (GTX26131, 1:1000 for western blot) was purchased from GeneTex. Rabbit anti-fibrin/fibrinogen polyclonal antibody (A0808, 1:2000 for IHC staining) was obtained from Dako/Agilent. FITC-conjugated anti-mouse P-selectin antibody was purchased from Pharmingen (553744). Mouse cytokines IL-6, TNFα, and IL-10 ELISA kits were obtained from R&D Systems (Minneapolis, MN). The DetectX Urea Nitrogen (BUN) Detection Kit was purchased from Arbor Assays (Ann Arbor, MI). Crea- and alanine transaminase coloform assay kits were from Cayman Chemical (Ann Arbor, MI). Mouse Cystatin C ELISA kit was from Theradiosfilter Sciences (Waltham, MA). Mouse thrombin-antithrombin complexes ELISA kit (TAT, ab137994) and mouse fibrinogen ELISA kit (ab213478) were obtained from abcam (Cambridge, MA). Mouse D-Dimer (D2D) ELISA kit (EKC6716) was purchased from Biomatik (Wilmington, DE). Rabbit anti-VWF antibody (AB7356) and Poly (D-lysine methylacrylate) gel from (192060) was purchased from Millipore-Sigma (Kankakee, IL). PE/Cyanine7 labeled rat anti-mouse Ly-6G Antibody (clone 1A8, 127618) was purchased from Biolegend (San Diego, CA).

**HLPN preparation.** MB2mP6 (Myr-FEKEKL) and scrambled control (Myr-EFK-KLE) peptides were synthesized and purified by the Research Resources Center at the University of Illinois at Chicago or custom-made by CPC Scientific (San Jose, CA) in some experiments. PEG2000-DSPE (Avanti Polar Lipids Inc. Alabaster, AL), L-α-phosphatidylincholine (egg PC, Type XI-E, Sigma-Aldrich, St. Louis, MO) and peptides were mixed at a molar ratio of 55:6:34:38. Our data showing elevation of pro-inflammatory cytokines, IL-6 and TNFα as well as the hypercoagulant state at 6 h and elevation of late anti-inflammatory cytokine IL-10 and D-dimer at 18 h, are consistent with these reports. Thus, MB2mP6 is not purely preventative, but rather disrupts the progression of systemic inflammation. However, anti-inflammatory/anti-thrombotic therapy will likely be successful if used before the grave “suppression” phase of the disease. If the patients/experimental animals have succumbed to the consequences of inflammation and thrombosis (e.g., multiple organ failure), they are unlikely to be revived with these (or likely any other) drugs. Indeed, the start of the treatment with MB2mP6/antibiotics immediately after sepsis onset resulted in better survival compared to infusion 6 or 18 h after CLP. Thus, the use of this type of drug early is likely to be more effective in disrupting the progression of systemic inflammation into the irreversible phase.

**Cell culture and differentiation of THP-1 cells.** THP-1 cells (ATCC TIB-202) were maintained in RPMI-1640 supplemented with fetal bovine serum (10%); 2-mercaptoethanol (0.5 mM); HEPES (10 mM), sodium pyruvate (1 mM), penicillin, and streptomycin (100 μg/mL). Cells were grown to a density of 6–8×106 cells mL−1 and subjected to subculturing or seeding by the Research Resources Center at the University of Illinois at Chicago or custom-made by CPC Scientific (San Jose, CA) in some experiments. PEG2000-DSPE (Avanti Polar Lipids Inc. Alabaster, AL), L-α-phosphatidylincholine (egg PC, Type XI-E, Sigma-Aldrich, St. Louis, MO) and peptides were mixed at a molar ratio of 55:6:34:38. HLPN were prepared using a film rehydration method.

**Co-immunoprecipitation.** Co-immunoprecipitation of integrin β3, or β3 and Gα13, was performed similarly to the previously described procedure. Brieﬂy, differentiated THP-1 cells (2×106) or human platelets (5×1010/mL) were incubated with MB2mP6 HLPN (50 μM) or scrambled peptide HLPN (50 μM). Ten min after treatment, THP-1 cells or platelets were stimulated with LPS (100 ng mL−1) or thrombin (0.025 U mL−1) for various time points and solubilized with NP40 lysis buffer (50 mM Tris, pH 7.4, 10 mM MgCl2, 150 mM NaCl, 1% NP-40, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF) with complete protease inhibitor cocktail tablets (Roche). After centrifugation at 17,800×g for 10 min at 4 °C, lysates were then collected and immunoprecipitated with mouse anti-integrin β3 antibody (1.BB.246, 2 μg per 500 μg lysate) or mouse anti-integrin β1 IgG (M15, 2 μg per 500 μg lysate) and an equal amount of mouse IgG (overnight at 4 °C) and then 100 μM protein A/G plus agarose beads (sc-2003, Santa Cruz Biotechnology, Inc, Dallas, TX) for 1 h at 4 °C. Following three washes with NP40 lysis buffer, immunoprecipitates were analyzed by western blot.
Detection of cytokine expression in THP-1 cells and mouse macrophages. PMA-differentiated THP-1 cells were collected using 10 μM EDTA in PBS and seeded onto six-well plates for 14 h. After serum starving for 4 h, the THP-1 cells were stimulated with LPS for 8 h. The total RNA was purified from the THP-1 cells using a TRIzol reagent (Invitrogen). Following purification, the RNA was treated with RNase-free DNase I (Invitrogen) to remove any contaminating genomic DNA. The treated RNA was reverse transcribed using random hexamers and Superscript III (Invitrogen) to generate cDNA. The resulting cDNA was then amplified using specific primer sets. The primers were designed to amplify a 100 bp fragment of the human NF-κB p65 gene. The PCR products were then separated on a 2% agarose gel and visualized under UV light. The results showed that the THP-1 cells expressed the NF-κB p65 gene at a high level.

THP-1 cell adhesion assay. To test the adhesion of differentiated THP-1 cells on human ICAM-1 surface, we precoated the 48-well plates with recombinant hICAM-1 (10 μg/mL). The THP-1 cells were stained with 0.5% trypan blue and allowed to adhere for 30 or 60 min. The non-adherent cells were washed away with PBS. The adherent THP-1 cells were stained with 0.5% trypan blue and counted using a hemocytometer.

Platelet preparation, aggregation, and granule secretion. 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.

Fibrinogen binding assay. To measure fibrinogen binding, 3 x 10^11 mL −1 washed mouse platelets in modified Tyrode’s buffer containing 0.1% bovine serum albumin (BSA) (pH 7.4) were incubated with fibrinogen (1 mg/mL) for 10 minutes. The platelets were then washed and resuspended in modified Tyrode’s buffer. The platelets were then incubated with FITC-conjugated rat anti-mouse P-selectin antibody (BD Pharmingen, 553744) for 30 min at room temperature. After dilution 10 times in PBS (with 1%BSA), P-selectin expression was analyzed using a Accuri C6 flow cytometer.

Neutrophil phagocytosis and bacterial killing assays. Mouse neutrophil phagocytosis was performed as previously described. Mouse neutrophils were isolated from the bone marrow of C57 wild type mice and resuspended in RPMI 1640 medium containing 10% FBS. After resting at 37 °C for 30 min, the neutrophils (2 x 10^11 mL −1 ) were seeded onto six-well plates and pretreated with MB2mP6 or scrambled peptide HLPN in RPMI 1640 cell culture medium for 20 min and loaded into ICAM-1 precoated 48-well plates (200 μL). After allowing cells to adhere for 30 or 60 min, the cell medium was aspirated, and the non-adherent cells were washed away with PBS. The adherent cells were stained with 0.5% trypan blue and counted using a hemocytometer.

Mouse jugular vein cannulation and continual peptide infusion. The jugular vein was cannulated using a 22-gauge needle. The catheter was then advanced into the right jugular vein and secured with a 6-0 suture, without closing off the catheter. The wound was closed using a 6-0 suture. The catheter was then connected to a heated platform under a dissection microscope. A 5 mm incision through the skin on the upper part of the mouse back was made posterior to the ears and between the scapulae. The mouse was then placed in a supine position and the skin over the right-side jugular vein was shaved and cleaned with hexachlorophene and 70% ethanol. After a vertical incision over the jugular site, the vein was dissected and isolated. The catheter was then advanced into the jugular incision through a trocar sleeve kit. The jugular vein was carefully separated, and two sutures were drawn around the vessel and tied loosely. Then, a small incision was made in the vein, the beveled catheter was inserted and tied in place with a suture, without closing off the catheter. The wound was closed using a 6-0 suture and a collagen fibrin sealant (Technic). For peptide treatment, MB2mP6 or control peptide micelles were injected in a bolus dose of 2.5 μg kg −1 followed by a continuous infusion at a rate of 1.25 μg kg −1 h −1 for 5 days.

FeCl3-induced mouse carotid artery thrombosis model. Eight- to 10-week-old C57BL/6 mice (20–25 g body weight) were anesthetized by isoflurane inhalation. The right carotid artery was isolated. A MA-0.5SB nano probe (Transformic, Ithaca, NY) was hooked to the carotid artery to monitor blood flow with a TS420 flowmeter (Transonic Systems). After dilatation, a filter paper disc (2 mm diameter) soaked with 1.2 μL of 7.5% FeCl3 (Sigma Aldrich, St. Louis, MO) was placed at the tip of the carotid artery for 3 min to induce thrombosis and then removed. MB2mP6 or scrambled control peptide were injected 15 min before the procedure retro-orbitally. Blood flow was monitored continuously until 5 min after occlusion or for 15 min if no occlusion occurred. Time to occlusion was defined as the time between the removal of the filter paper and stable occlusion (no blood flow at the carotid artery). The carotid arteries were then collected and compared medians between treatments by Graphpad Prism software (version 8.3.0).

Tail bleeding time. Tail bleeding time was performed as previously described. Eight- to 10-week-old C57BL/6 mice weighing 20–25 g were anesthetized with isoflurane. After cutting a 0.5-cm-long segment off the distal tip of the tail, their tails were immersed in 0.15 M NaCl immediately at 37 °C. Tail bleeding time was defined as the time between cutting the tail and stable cessation of bleeding (no bleeding within 60 s). Bleeding was observed for up to 15 min. If bleeding persisted at 15 min, bleeding was stopped by application of pressure. Data were analyzed using the Mann–Whitney test to compare medians between groups by GraphPad Prism software (version 8.3.0).

rpA reaction. C57BL/6 mice were anesthetized by i.p. injection of a mixture of ketamine and xylazine (100 mg kg −1 and 16 mg kg −1 mouse weight), and the same volume of the mouse was shaved. Shaved mice were injected with i.v. injection of rabbit anti-RSA antibody (6 μg mL −1.1 MP Biomedical, 0.25 mL of PBS, followed by i.v. injection of RSA (75 μg g −1 mouse weight) as well as the peptide inhibitors (5 μmol kg −1 mouse weight) in 100 μL sterile 0.9% NaCl. In addition, control sites received 25 μL of PBS in parallel. Four hours after injection, mice were euthanized and inflamed or control skin samples at each injection site were collected. The HB contents in the skin samples were quantified by Hb colorimetric assay kit (Cayman Chemical).

CLP sepsis model. CLP sepsis was induced as described previously. In brief, mice (14- to 16-week-old with an equal number of each gender) were anesthetized by intraperitoneal administration of ketamine (100 mg kg −1 body weight) and xylazine (8 mg kg −1). After a midline laparotomy, the cecum was ligated at about one third of the cecum and then subcutaneously injected with a 18-gauge needle. Sham-operated mice underwent the same procedure except for ligation and puncture of the cecum. After the procedure, an analgesic (buprenorphine, 0.1 mg kg −1, s.c.) was given immediately and every 12 h thereafter for 3 d. At these time points, fluid resuscitation (prewarmed 0.9% NaCl, 0.9% NaCl with saline and double bolus administration with an 18-gauge needle) was started at the normal rat weight except for ligature and puncture of the cecum. After the procedure, the anaerobic (Claronat solution; 10 μg kg −1 body weight) was subcutaneously administered (to mimic clinical conditions) at the time MB2mP6 treatment was initiated and lasted for 5 days. The medication was continued until the situation of scheduled sacrifice, death, or study completion in the surviving mice. The survival of mice was observed every 6 h for 8 days and analyzed by the Log Rank test (GraphPad Prism software, San Diego, CA). For CLP-induced mouse organ injury, blood samples and kidney or lung tissues were collected 24 h after CLP and subjected to cytokine and immunohistochemistry analyses.
Measurement of organ injury. Twenty-four hours after CLP, mouse blood was collected and added to an EDTA-rinsed microcentrifuge tube or citrate buffer. The plasma was obtained after centrifugation at 1000 g × 4°C for 5 min. In some cases, mouse serum was collected from blood without adding any anti-coagulant. The kidney function markers BUN, creatinine and cystatin C and mouse coagulant factors fibrinogen and TAT were detected using corresponding kits according to the manufacturers’ instructions. Septic mouse kidney, lung, and liver tissue were collected from the sacrificed mice 24 h after CLP, as well as from sham-operated mice and fixed in 10% formalin.

Immunohistochemistry. Mouse kidney and lung were collected 24 h after CLP after perfusion with phosphate-buffered saline and then fixed in 10% formalin solution. Twenty hours after fixation, mouse tissues were dehydrated in 70% ethyl alcohol and embedded in paraffin. Four to five-micrometer sections were cut and stained with hematoxylin and eosin for quality control. Sections were de-paraffinized, rehydrated, peroxidase blocked, and subjected to antigen by heating at 95°C for 20 min in pH 6.0 citrate buffer. Rat anti mouse integrin αIIb antibody (1:250, Clone MWReg 30), rabbit anti fibrin/fibrinogen antibody (1:2000, A0080, Dako), rabbit anti-VWF antibody (1:50, A87356, Milipore Sigma) and anti-tat or anti-rabbit IgG-avidin-biotin complex kits (Vector Laboratories) were used to stain platelet-rich or fibrin-rich thrombi. The positive stain in each section was quantified using ImageJ software. Slides were also stained with Mallory’s PTAH (PTAH Stain kit, American MasterTech, McKinney, TX) to identify fibrin deposition and viewed with a Leica DMI RB microscope using a 40 × 0.55 NA objective. Total thrombotic area/glomerulus was quantitated by analyzing 20–30 glomeruli from each group using ImageJ software.

Detection of cytokine expression. Twenty-four hours after CLP, mouse blood was collected from sham-operated and CLP-induced septic mice. The serum was isolated and analyzed for cytokine levels using specific ELISA kits. Mouse lungs were also collected from those sacrificed mice after PBS perfusion. Cytokine transcripts expressed in lung tissue were detected by real-time PCR using SYBR green method.

Mouse lung microvascular permeability assay. Mouse lung microvascular permeability was determined as described previously. Briefly, mice were i.v. injected with Evans blue albumin (EBA, Sigma, 25 mg kg⁻¹) 30 min before the termination of CLP or sham operation to assess vascular leakage. After 30 min, the lungs were perfused with PBS and excised out of the thoracic cavity. After homogenization in PBS (1 mL per 100 mg of lung tissue), lungs were further incubated in two volumes of formamide (Sigma) for 18 h at 65°C. The concentration of Evans blue present in each milligram of lung tissue was measured immediately by a luminometer (Femtomaster FB 12, Zylux Corporation).

Statistical analysis. Data are expressed as means ± SEM. For parametric data, differences between groups of samples were evaluated with student’s t-test, one-way ANOVA, or two-way ANOVA with GraphPad Prism software. For non-parametric data, statistical significance was determined using the Mann-Whitney test. Survival analysis was performed using GraphPad Prism software with the Kaplan-Meier method. A p-value ≤ 0.05 was considered statistically significant.

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Competing interests N.C. has ownership interests in DMT Inc., which licenses the university patents. R.A.S. is employed by DMT Inc. The other authors declare no competing interests.

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