Endotoxemia is often associated with extreme inflammatory responses and disseminated intravascular coagulation. Protease-activated receptors (PARs) mediate cellular responses to coagulation proteases, including platelet activation and endothelial cell reactions predicted to promote inflammation. These observations suggested that PAR activation by coagulation proteases generated in the setting of endotoxemia might promote platelet activation, leukocyte-mediated endothelial injury, tissue damage, and death. Toward testing these hypotheses, we examined the effect of PAR deficiencies that ablate platelet and endothelial activation by coagulation proteases in a mouse endotoxemia model. Although coagulation was activated as measured by thrombin-antithrombin (TAT) production and anti-thrombin III (ATIII) depletion, Par1−/−, Par2−/−, Par4−/−, Par2−/−:Par4−/−, and Par1−/−:Par2−/− mice all failed to show improved survival or decreased cytokine responses after endotoxin challenge compared with wild type. Thus, our results fail to support a necessary role for PARs in linking coagulation to inflammation or death in this model. Interestingly, endotoxin-induced thrombocytopenia was not diminished in Par4−/− mice. Thus, a mechanism independent of platelet activation by thrombin was sufficient to cause thrombocytopenia in our model. These results raise the possibility that decreases in platelet count in the setting of sepsis may not be caused by disseminated intravascular coagulation but instead report on a sometimes parallel but independent process. (Blood. 2006;107:3912-3921)

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Roles of protease-activated receptors in a mouse model of endotoxemia

Eric Camerer, Ivo Cornelissen, Hiroshi Kataoka, Daniel N. Duong, Yao-Wu Zheng, and Shaun R. Coughlin

Endotoxemia is often associated with extreme inflammatory responses and disseminated intravascular coagulation. Protease-activated receptors (PARs) mediate cellular responses to coagulation proteases, including platelet activation and endothelial cell reactions predicted to promote inflammation. These observations suggested that PAR activation by coagulation proteases generated in the setting of endotoxemia might promote platelet activation, leukocyte-mediated endothelial injury, tissue damage, and death. Toward testing these hypotheses, we examined the effect of PAR deficiencies that ablate platelet and endothelial activation by coagulation proteases in a mouse endotoxemia model. Although coagulation was activated as measured by thrombin-antithrombin (TAT) production and anti-thrombin III (ATIII) depletion, Par1−/−, Par2−/−, Par4−/−, Par2−/−:Par4−/−, and Par1−/−:Par2−/− mice all failed to show improved survival or decreased cytokine responses after endotoxin challenge compared with wild type. Thus, our results fail to support a necessary role for PARs in linking coagulation to inflammation or death in this model. Interestingly, endotoxin-induced thrombocytopenia was not diminished in Par4−/− mice. Thus, a mechanism independent of platelet activation by thrombin was sufficient to cause thrombocytopenia in our model. These results raise the possibility that decreases in platelet count in the setting of sepsis may not be caused by disseminated intravascular coagulation but instead report on a sometimes parallel but independent process. (Blood. 2006;107:3912-3921)

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Introduction

Endotoxemia often triggers exuberant inflammatory responses and activation of the coagulation cascade, and interactions between inflammation and coagulation may be important in this setting. The importance of inflammatory responses to endotoxin as a determinant of survival and a role for inflammatory mediators in promoting coagulation seem clear from studies in animal models of sepsis. Endotoxin and the inflammatory cytokines that it induces can cause expression of tissue factor (TF), the trigger for coagulation, by circulating monocytes and by cells outside the vascular compartment, and endothelial damage from leukocyte products can allow circulating coagulation factors access to extravascular TF and impair natural anticoagulant pathways. These events can trigger disseminated intravascular coagulation (DIC) and, importantly, antibodies to inflammatory cytokines can both prevent DIC and improve survival in animal models of sepsis.

The roles and relative importance of coagulation as a regulator of inflammatory responses to endotoxin and survival in endotoxemia are less clear. There are many potential mechanisms by which coagulation might modulate inflammatory responses. In cell culture, proinflammatory as well as cytoprotective cellular responses to coagulation factors have been described. In a baboon model of Escherichia coli sepsis, some inhibitors of coagulation (anti-TF antibodies, active-site inhibited factor VIIa [VIIai] and TF pathway inhibitor [TFPI]) reduce cytokine levels and improve survival, while other inhibitors (Xai) prevent DIC but do not affect cytokine responses or survival. In a mouse model of E coli sepsis, inhibition of TF/VIIa did not affect inflammation, but mice with low TF expression did show a decreased cytokine response to endotoxin. Activated protein C (APC), an endogenous anticoagulant that is activated by thrombin, is protective in sepsis, but this effect may be due to activities of APC that are unrelated to its anticoagulant function. In mouse, PAR4 is necessary for platelet activation by thrombin, and PAR2 can be activated by coagulation proteases VIIa and Xa but not thrombin. In mouse, PAR4 is necessary for platelet activation by thrombin; PAR1 is the main thrombin receptor on microvascular endothelial cells and mesenchymal cells, and PAR1 and PAR2 together account for TF/VIIa and Xa signaling in these cells. PAR1 is necessary for thrombin responses in smooth muscle cells and fibroblasts. Accordingly, we used mice deficient in PAR1, PAR2, PAR4, both PAR1 and PAR2, or both PAR2 and PAR4 to probe the importance of responses to coagulation proteases in platelets, endothelial cells, and other cells in a model of endotoxemia.

From the Cardiovascular Research Institute, University of California, San Francisco.


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Reprints: Shaun Coughlin, University of California, San Francisco, HSE-1307, 513 Parnassus Ave, San Francisco, CA 94143-0130; e-mail: shaun.coughlin@ucsf.edu.

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Materials and methods

Mouse lines

C57BL/6 mice were purchased from Jackson Laboratory (JAX, Bar Harbor, ME). Genes encoding mouse PAR1, PAR2, and PAR4 are designated Par1, Par2, and Par4, respectively; alternative names are F2r, F2rl1, and F2rl3, respectively. Generation of Par1−/−, Par2−/−, and Par4/−/− mice has been described.38,43,44 Par2−/−:Par4−/− mice were generated from intercrosses of single-deficient mice. Par1−/+;Par2−/− mice were generated by double targeting of the PAR locus on mouse chromosome 13 (E.C. and S.R.C., manuscript in preparation). Fibrinogen-deficient (Fib−/−) mice44 and Nf-E2−/− mice45 were generously provided by Drs Jay L. Degens (Children’s Hospital, Cincinnati) and Stuart Orkin (Harvard Medical School), respectively. Par1, Par2, Par4, and Par2:Par4 lines had been backcrossed 5 to 6 generations into the C57BL/6J strain. Due to low survival rates in the C57BL6 background, Par1−−−;Par2−−−, Par2−−− (littermate study), Fib−/−, and Nf-E2−/− lines were maintained in a 129/SVJ-C57BL6 mixed background (referred to as 129/B6). Initial studies of Par1−/−, Par2−/−, and Par4−/− mice were done in the C57BL6/ background and compared with mice generated by homzygous (−/−) or wild-type (+/+) intercrosses and matched for age and sex. Follow-up studies used littermates from heterozygote (+/−) crosses. High embryonic lethality (95%) of Par1−−−:Par2−−− mice precluded us from using this strategy for these mice; Par1−−−;Par2−−− mice were generated only by Par1−−−: Par2−−− “survivor” intercrosses and compared with age- and sex-matched wild-type mice in the same strain background from the same colony (ie, derived from previous Par1−−−;Par2−−− intercrosses). Fib−/− and Nf-E2−/− mice were generated by +/− intercrosses.

Endotoxin survival studies

Protocols were approved by the Committee on Animal Research at the University of California, San Francisco. Mice 8 weeks of age or older were injected intraperitoneally with the specified dose of endotoxin (also referred to as lipopolysaccharide [LPS]; serotype 0111:B4; Sigma, St Louis, MO) in normal saline or with saline alone and monitored either every 2 hours from 12 to 26 hours after injection (high-dose protocol) or every 12 hours for 3 days after injection (low-dose protocol). With the exception of a few hirudin-treated animals that died early from intraabdominal bleeds and were excluded from the study, no deaths were observed in the first 12 hours after LPS administration. Mice without a righting reflex at the time of injection were included in the survival curves and accounted for approximately 20% of all deaths. Recombinant hirudin (Refudan; kindly provided by Berlex Biosciences, Richmond, CA) or saline control was injected intraperitoneally 30 minutes before to endotoxin. Antiplatelet serum (100 μL of a 1:10 dilution; Inter-cell Technologies, Hopewell, NJ) or control serum was injected intraperitoneally 24 hours prior to and 24 hours after endotoxin. A single injection of 100 μL of a 1:10 dilution of antiplatelet serum resulted in more than 90% reduction in platelets for at least 48 hours by manual counts. The antiserum also reduced levels of most white cell populations. Higher doses or more frequent injections of antiserum led to anesthesia and sometimes death.

Short-term effects of endotoxin and PAR agonists

To study biochemical and cellular markers of inflammation and coagulation, mice were injected with endotoxin or PAR agonists and blood samples were obtained at different time points. Blood was obtained from the retro-orbital plexus before treatment or at intermediate time points, and for terminal samples blood was drawn from the inferior vena cava (IVC) of anesthetized mice into EDTA-coated tubes or into 1:10 volume sodium citrate. Perfusion with normal saline followed by 10% neutral buffered formalin was performed when organs were to be collected for histology. Complete blood counts (CBCs) were determined using a Hemavet 850FS (DREW Scientific, Oxford, CT). Hemavet platelet counts were comparable to manual hemocytometer counts done in parallel for counts in the range of 100 × 10³/μL (300 × 10³/μL to 1000 × 10³/μL, 1000 × 10³/μL but diverged when counts were below 200 × 10³/μL (200 000/μL), with Hemavet exceeding manual counts. For consistency, Hemavet counts are shown and differences noted where appropriate. Plasma samples were frozen and later analyzed for thrombin-antithrombin (TAT; Enzygnost TAT assay; Becton Dickinson, San Jose, CA) and cytokines (R&D Systems, Minneapolis, MN) by enzyme-linked immunosorbent assay (ELISA). Antithrombin III (ATIII) levels were determined using the AMAX Accucolor ATIII kit (Trinity Biotech, St Louis, MO). The kit uses a chromogenic substrate to measure how efficiently exogenous bovine thrombin is inhibited by diluted sample plasma in the presence of heparin. ATIII levels are given as a percentage of a normal value determined using pooled plasma from untreated mice.

Regarding the cytokine assays, we chose to focus on IL-6, IL-10, IL-8, and MCP-1 because (1) cytokine production dependent on protease signaling would be expected to occur secondary to TF induction and therefore relatively late after LPS challenge, and altered cytokine profiles by inhibitors of coagulation in various models of LPS- and E coli–induced systemic inflammation had suggested that coagulation might regulate these “late” responders but not “early” responders such as TNFs and IL-1β,123 and (2) IL-8 and MCP-1 are known to be induced by PAR1 activation in cultured endothelial cells.47,50 Regarding the TAT assay, it is notable that the decreased TAT levels measured in mice treated with hirudin might well be due to occupancy of thrombin’s active center by hirudin and do not necessarily imply decreased thrombin generation. However, decreased TAT formation should still reflect blockade of thrombin’s active site, the goal of hirudin treatment.

Phosphoinositide hydrolysis

Endothelial cells were immunopurified from the skin of Par1−/−:Par2−/− neonates or strain-matched wild-type controls as described.40 Cells in 24-well plates were loaded overnight with 2 μCi/mL (72 kBq/well) myo-[3H]inositol in DMEM/bovine serum albumin (0.2%) without serum. Cells were washed in phosphate-buffered saline, changed to fresh DMEM/bovine serum albumin without myo[3H]inositol for 2 hours, treated with 20 mM LiCl in DMEM/bovine serum albumin with or without agonist for 90 minutes, and then extracted with formic acid. Released total [3H]inositol phosphates were quantitated as described.51

Immunohistochemistry

Paraffin-embedded organs from saline- and formalin-perfused animals were sectioned at 5 μm and immunostained with rabbit antifibrinogen (DakoCytomation, Glostrup, Denmark) or anti–mouse platelet serum (Inter-cell Technologies) followed by a peroxidase-conjugated secondary antibody to rabbit IgG. Peroxidase activity was detected using a substrate kit from Vector Laboratories (Burlingame, CA), and tissues were counterstained with nuclear fast red. Spot software from Diagnostic Instruments (Burlingame, CA) was used to acquire images from a Nikon Diaphot-FXA microscope (Nikon, Tokyo, Japan). All images shown were visualized with a Nikon PlanApo 20 × 0.75 objective lens.

Statistical analysis

Survival curves were compared using the log-rank test with GraphPad Prism software. Cytokines and cell counts were compared using a Student t test or analysis of variance (ANOVA) where appropriate. Analysis of statistical power and correction for multiple comparisons was performed as described.52
Results

Choice of endotoxin dose and effect of sex on mortality rates

Our experiments were designed to detect either exacerbation or amelioration of responses to endotoxin and used different doses of endotoxin to yield different survival rates and syndromes that evolved with different tempos. In a wild-type C57BL/6 background, high-dose (60 mg/kg) endotoxin produced a rapid response and resulted in an approximate 10% overall survival at 26 hours and, interestingly, a higher mortality rate in males than in females (Figure 1). Thus, high-dose endotoxin produced a response in which any improvement in survival in mutant mice would be easily detected. It also revealed a sex difference in sensitivity to endotoxin in C57BL/6 mice that was also seen at lower endotoxin doses. We adjusted for this differential sensitivity to endotoxin in the low-dose study; males received 20 mg/kg and females 30 mg/kg. At these doses, survival in both males and females was about 40% at 72 hours (Figure 1), a situation in which either increased or decreased survival might be detectable. All subsequent experiments employed a “high dose” of 60 mg/kg for males and females and a “low dose” of 20 mg/kg for males and 30 mg/kg for females and, except in Figure 3C, we conducted all comparisons within a single sex to minimize variability. Note that “high dose” and “low dose” are convenient shorthand; the “low” dose was above the LD50 at 72 hours.

Characterization of disseminated intravascular coagulation

To characterize systemic activation of coagulation in our model, we examined TAT and ATIII levels and microvascular fibrin deposition after endotoxin exposure (Figure 2). Plasma TAT levels rose from about 10 ng/mL before endotoxin treatment to 40, 70, and 100 ng/mL at 4, 8, and 12 hours after low-dose endotoxin, respectively. The ATIII level was about 90% of normal at 4 hours but fell to 30% and 20% of normal by 8 and 12 hours, respectively. Platelet counts fell from 970 × 10^9/L to 480 × 10^9/L, 310 × 10^9/L, and 270 × 10^9/L at 4, 8, and 12 hours after endotoxin, respectively (however, subsequent studies suggested that such decreases were unrelated to thrombin signaling [see “PAR4 deficiency and endotoxin-induced thrombocytopenia”]). Similar results were obtained when high-dose endotoxin was employed, but the drop in ATIII levels at the later time points was larger than that seen with low-dose endotoxin. Most importantly, there was obvious fibrin staining in the microvasculature of the liver (Figure 2) and, to a lesser extent, kidney and lung (not shown) at 8 hours, and this was prevented by treatment with the thrombin inhibitor hirudin. Overall, these results suggest that coagulation was activated in our model to a degree sufficient to deplete ATIII and cause intravascular fibrin deposition in tissues. Thus, the model provided a setting in which activation of PARs on cells exposed to plasma might be expected to occur.

Survival of endotoxin exposure in mice with single PAR deficiencies

We next examined the effect of deficiencies of single PARs on survival after high- or low-dose endotoxin by comparing mice derived from intercrosses compared with age-, strain-, and sex-matched wild-type controls derived from the same colony. A comparison of mice lacking PAR1, PAR2, or PAR4 with matched wild-type controls, all crossed 5 to 6 generations into C57BL/6, did not reveal dramatic increases or decreases in survival in any group (Figure 3A). However, Par2−/− females at high-dose endotoxin appeared to show some protection, and male and female Par1−/− mice and Par4−/− females showed a trend toward apparent increased sensitivity to low-dose endotoxin. Three of these comparisons yielded an independent P value of less than .05, but with 12 comparisons, the probability that this level of significance would be achieved by chance at least once is about 50%. No comparison was significant at the Bonferroni-adjusted P value of .004.

Although typical of mouse endotoxemia experiments, the studies shown in Figure 3A had a power of only 50% to 70% for detecting a 30% increase in survival, and use of the Bonferroni adjustment in this setting and for this many comparisons is likely too stringent. Moreover, although the comparisons above were made in relatively inbred backgrounds and used control mice from the same breeding colonies, it is possible that chance residual differences in genetic backgrounds may have obscured positive
Addressing redundancy

Several intriguing hypotheses regarding the mechanisms by which coagulation might modulate inflammation and survival in endotoxemia focus on endothelial cells (see “Discussion”). PAR1 and PAR2 are both expressed on endothelial cells and can signal in response to multiple coagulation proteases (factor VIIa, factor Xa, thrombin, and APC) at least in vitro. Thus, the apparent lack of phenotype in mice individually deficient in PAR1 or PAR2 might be due to partially redundant roles for these receptors. Indeed, Pawlinski and colleagues recently reported that treatment with the thrombin inhibitor hirudin uncovered genotype-dependent protection of Par2−/− mice from endotoxin, consistent with the hypothesis that elimination of both PAR1 and PAR2 signaling might be important.3 However, hirudin treatment also prevents thrombin from cleaving substrates other than PAR1 including fibrinogen, and elimination of fibrinogen by ancrd treatment was shown to be protective in the same study.4 To address the question of possible redundancy among PARs more directly, we generated mice with combined PAR deficiencies. Embryonic lethality (E.C. and S.R.C., manuscript in preparation) precluded us from using Par1−−:Par2−−: Par4−− mice in this study. Embryonic lethality was also problematic in Par1−−:Par2−− mice, but in a mixed 129Sv/C57BL6 background adequate numbers of these mice could be generated for limited studies aimed at probing the importance of endothelial responses to coagulation proteases. Endothelial cells isolated from Par1−−:Par2−− mice were almost completely insensitive to all PAR-activating proteases as assessed by agonist-induced phosphoinositide hydrolysis (Figure 4). Only weak and variable responses to high concentrations of thrombin were seen, likely attributable to low and variable expression of PAR4 in such cells.40 Factor Xa and APC were incapable of triggering phosphoinositide hydrolysis in Par1−−:Par2−− endothelial cells, consistent with a previous study of endothelial cells from single knockouts suggesting

Figure 3. Survival of PAR-deficient mice after endotoxin challenge: single knockouts. (A) Par1−/− (top panels), Par2−/− (middle panels), and Par4−/− (bottom panels) mice and strain- (about 97.5% C57BL/6, about 2.5% 129SvJae), age-, and sex-matched controls were injected intraperitoneally with LPS and monitored for 26 (high-dose LPS) or 72 (low-dose LPS) hours. (B) Comparisons that showed trends toward differences in panel A were repeated in littermate-controlled studies. These were in the same strain background as in panel A, except for Par2, which was 50% C57BL/6, 50% 129SvJae. (C) Pooled low-dose data from panels A-B. The top panel compares wild-type mice in this study. Embryonic lethality was also problematic in Par1−−:Par2−− mice. In all panels, high-dose LPS was 60 mg/kg for both sexes; low dose was 30 mg/kg for females and 20 mg/kg for males. These dosing conventions were maintained for all subsequent studies. P values as determined by log-rank test are shown when < .05.
zymes and candidate PAR-activating proteases that might be present in response to high-dose endotoxin (Figure 5A; Table 1). However, endothelial cells. Tryptase and proteinase 3, both leukocyte gran-

Figure 5. Survival of PAR-deficient mice after endotoxin challenge: double
knockouts. (A) Par1+/−:Par2−/− (50% C57BL/6, 50% 129SvJae) mice and strain-

Cytokine levels and circulating cell counts after endotoxin in PAR-deficient and other mice

Individual deficiency of PAR1, PAR2, PAR4, or fibrinogen was not associated with significant changes in plasma levels of TAT, IL-6, MIP-2, MCP-1, or IL-10 (Table 1) in peripheral cell counts (Table 2) 8 hours after endotoxin treatment. In accord with these results, we did not see differences in the histology of liver, kidney, and lung between knockouts and wild-type controls (not shown). Treatment with hirudin sufficient to cause a more than 90% reduction in TAT levels and a drop in hematocrit also failed to effect cytokine production in response to endotoxin as did treatment with antiplatelet antiserum sufficient to cause an 80% to 90% reduction in manual platelet counts. Cytokine levels trended higher, not lower, in Par1+/−:Par2−/− mice but were within the range seen in control groups across multiple experiments, and Par2−/− mice treated with hirudin did not show decreases in IL-6 levels. Peripheral leukocyte counts trended lower in Par1+/−:Par2−/− mice but again not outside the range seen in controls in other experiments, and lower cell counts were not found in Par2−/− mice treated with hirudin.

PAR4 deficiency and endotoxin-induced thrombocytopenia

Endotoxin reliably caused thrombocytopenia in wild-type mice, and we hypothesized that activation of platelets by thrombin produced in association with DIC would contribute to thrombocytopenia in this model. Interestingly, thrombocytopenia was not diminished in Par4−/− mice despite the absence of thrombin-mediated platelet activation in these mice (Figure 6). In accord with equally decreased platelet counts, platelet accumulation in the liver microvasculature appeared similar in Par4−/− mice and the littermate controls (Figure 6). A repeat of this study using high-dose endotoxin also revealed no significant difference in the decrease in platelet counts in wild-type and Par4−/− mice. At 12 hours, a time at which ATIII had been largely consumed and TAT was elevated (Figure 2), platelet counts were 307 × 10^9/L ± 92 × 10^9/L in Par4−/− versus 233 × 10^9/L ± 98 × 10^9/L in wild-type sex-matched littermate controls (n = 4). Moreover, TAT levels were equally elevated in wild-type and Par4−/− mice (Table 1; data not shown). These results suggest that, in this model, thrombin signaling in platelets is not necessary for endotoxin-induced thrombocytopenia, and thrombin-induced platelet procoagulant activity is not important for thrombin generation.
studies, no decrease in cytokine levels was seen. TAT levels in hirudin-treated controls in other experiments. In the high-dose endotoxin across experiments (not shown), and cytokine levels in the low-dose endotoxin study, there was a trend toward reduction in cytokine levels in hirudin-treated mice, but it was not consistent. Again, no survival advantage was seen (Figure 7A). In the high-dose endotoxin exposure in mice treated with 5 mg/kg hirudin, TAT levels in mice not treated with endotoxin. were less than 10% of those seen in controls (Table 1) and were comparable to TAT levels in mice with ATIII and substrates. Accordingly, we repeated this experiment and activation of leukocytes and local edema.9,70 Given this evidence and prominence of DIC in sepsis syndrome, it has been tempting to envision a role for PARs as mediators of microvascular thrombosis and inflammation in response to endotoxin.3,11,13,36,71-74

### Effects of thrombin inhibition, fibrinogen deficiency, and platelet deficiency

Activation of coagulation clearly occurred in our model as evidenced by TAT formation, ATIII depletion, and microvascular fibrin deposition (Figure 2; Table 1). While not uniform, published studies have suggested a role for coagulation in promoting inflammation and death in mouse endotoxemia.3,25,60-62 The lack of protection in PAR-deficient mice led us to retest this notion by examining the effect of thrombin inhibition as well as fibrinogen and platelet deficiency in our system. Surprisingly, repeated injections of 1 mg/kg hirudin according to a published protocol3 did not improve survival or consistently decrease cytokine levels (Table 1; not shown). One milligram per kilogram of hirudin did not provide complete inhibition of TAT formation at 8 hours (Table 1), suggesting that some active thrombin was available for interaction with ATIII and substrates. Accordingly, we repeated this experiment at 5 mg/kg hirudin. TAT levels at 8 hours after low- or high-dose endotoxin exposure in mice treated with 5 mg/kg hirudin were less than 10% of those seen in controls (Table 1) and were comparable to TAT levels in mice not treated with endotoxin. Again, no survival advantage was seen (Figure 7A). In the low-dose endotoxin study, there was a trend toward reduction in cytokine levels in hirudin-treated mice, but it was not consistent across experiments (not shown), and cytokine levels in the hirudin-treated mice were still within the range seen in nonhirudin-treated controls in other experiments. In the high-dose endotoxin studies, no decrease in cytokine levels was seen.

Like thrombin inhibition, fibrinogen deficiency also did not provide protection (Figure 7B). Similarly, lack of circulating platelets associated with deficiency of Nf-E263 also did not protect against endotoxin-induced lethality (Figure 7C). On the contrary, there was a tendency for decreased survival of platelet-deficient mice compared with their littermate controls (Figure 7C). Similar results were obtained in mice made thrombocytopenic with antplatelet antibodies (Figure 7C). Most Nf-E2−/− mice did not show gross hemorrhage into brain, gut, lung, skin, or peritoneal or pericardial cavities at death, raising the possibility that increased mortality is due to a more subtle defect in microvascular integrity. Platelets are a rich source of sphingosine-1-phosphate (S1P), and S1P has been shown to be protective against endotoxin-induced lung injury.65 did not reverse the exacerbating effect of thrombocytopenia in endotoxemia; nor did it protect wild-type mice in this model (Figure 7C).

### Discussion

Activation of platelet PARs plays an important role in several models of thrombosis,38,66-69 and PARs on neurons and endothelial and mesenchymal cells in culture and in vivo can trigger recruitment and activation of leukocytes and local edema.9,70 Given this evidence and prominence of DIC in sepsis syndrome, it has been tempting to envision a role for PARs as mediators of microvascular thrombosis and inflammation in response to endotoxin.3,11,13,36,71-74 However, our study fails to support an important role for PARs in these processes in a mouse model of endotoxemia. No single or
combined PAR deficiency protected mice from inflammatory or lethal responses to endotoxin. This result was surprising to us in that TF deficiency was reported to be protective in a mouse model of endotoxemia as was combined PAR2 deficiency and thrombin inhibition.\(^3\) However, others have reported that inhibition of TF/VIIa failed to protect against inflammation or death in a mouse model of \textit{E coli} sepsis.\(^{25}\) Whether differences in the models themselves, in the inhibitors used, or in experimental design (eg, use of littermate controls, controlling for sex differences) account for such varied results is uncertain. Taken together, our results suggest that PAR activation does not play an important or necessary role in promoting inflammation and death in the mouse model of endotoxemia studied here. Our data of course do not necessarily imply that PARs play no role whatsoever in promoting inflammatory response. Endotoxin is a remarkably potent inducer of multiple cytokines. Thus, any proinflammatory contribution made by PARs might be redundant and unnecessary in endotoxemia but relatively more important in other settings.

Our finding that PAR4 deficiency did not diminish thrombocytopenia in response to endotoxin was somewhat surprising. Thrombocytopenia is often considered to be part of the consumptive coagulopathy associated with DIC induced by endotoxin, and we expected that thrombin generated in this setting would contribute to thrombocytopenia in response to endotoxin was somewhat surprising. Thrombin inhibition.\(^3\) However, others have reported that inhibition of TF/VIIa failed to protect against inflammation or death in a mouse model of \textit{E coli} sepsis.\(^{25}\) Whether differences in the models themselves, in the inhibitors used, or in experimental design (eg, use of littermate controls, controlling for sex differences) account for such varied results is uncertain. Taken together, our results suggest that PAR activation does not play an important or necessary role in promoting inflammation and death in the mouse model of endotoxemia studied here. Our data of course do not necessarily imply that PARs play no role whatsoever in promoting inflammatory response. Endotoxin is a remarkably potent inducer of multiple cytokines. Thus, any proinflammatory contribution made by PARs might be redundant and unnecessary in endotoxemia but relatively more important in other settings.

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Our finding that PAR4 deficiency did not diminish thrombocytopenia in response to endotoxin was somewhat surprising. Thrombocytopenia is often considered to be part of the consumptive coagulopathy associated with DIC induced by endotoxin, and we expected that thrombin generated in this setting would contribute to platelet aggregation and sequestration in the microvasculature. Platelets from \textit{Par4}–/– mice are unresponsive to even micromolar concentrations of thrombin,\(^{28}\) and the persistence of endotoxin-induced platelet accumulation in the microvasculature and thrombocytopenia in \textit{Par4}–/– mice (Figure 6) suggests that platelet activation by thrombin is not necessary for thrombocytopenia in
this model. Notably, the level of thrombocytopenia seen in our studies was relative (a 70% to 80% decrease in platelet counts); severe thrombocytopenia (less than 100 × 10^9/L) was not observed in our model even when mice were moribund. Thus, we cannot exclude a contribution of platelet activation by thrombin in situations in which severe thrombocytopenia occurs.

Like PAR4 deficiency, fibrinogen deficiency also failed to prevent thrombocytopenia (Table 2), suggesting that thrombocytopenia is not due to "trapping" of platelets by fibrin clots in the microvasculature. Moreover, increases in TAT were not different in Par4−/− mice and wild-type mice. Thus, endotoxin-induced DIC occurred in Par4−/− mice but did not cause thrombocytopenia, suggesting that decreases in platelet count may be a marker for another process. A recent study by Andonegui and colleagues suggests interesting possibilities. The endotoxin receptor TLR4 is expressed on platelets, and endotoxin can promote platelet adhesion to fibrinogen in a flow chamber assay. However, endotoxin does not by itself trigger P selectin display in isolated platelets, and in Tlr4−/− mice reconstituted with wild-type bone marrow and treated with endotoxin, platelets do not accumulate in the microvasculature. Thus, endotoxin acting on platelet TLR4 may contribute to platelet activation, but the major effects of endotoxin on platelets may be indirect. Interestingly, in this same study, neutrophil accumulation in the microvasculature preceded and was necessary for platelet sequestration, but platelets in lung microvasculature appeared to be interacting mainly with each other and with endothelium rather than with neutrophils. Moreover, at least in human platelets, PAR4 signaling is required for direct platelet activation by neutrophils in platelet-neutrophil suspensions, presumably because PAR4 can function as a receptor for the neutrophil granzyme cathepsin G. This system would also be eliminated in the Par4 knockout (if it even operates in mice). Taken together with our results, these observations suggest that thrombocytopenia in the setting of endotoxemia may not be a component of DIC but instead may reflect another process, perhaps one secondary to neutrophil accumulation in the microvasculature. These results also suggest that neutrophils may act indirectly to promote platelet activation by damaging or activating the endothelium and emphasize that the proximal activator of platelets in endotoxemia remains unknown.

What promotes neutrophil accumulation in the microvasculature? It might again be tempting to envision a role for PARs. Application of PAR2 or PAR4 agonists to exposed microvessels is sufficient to trigger rapid margination of leukocytes; intratracheal instillation of PAR2 agonist is sufficient to trigger edema and neutrophil accumulation in the airspace and lung interstitium in a receptor-dependent manner (apparently by an at least partly neuropeptide-dependent mechanism); and subcutaneous injection of PAR agonists triggers edema and neutrophil infiltration. However, combined deficiency of PAR1 and PAR2 or of PAR2 and PAR4 did not prevent decreases in circulating neutrophil counts after endotoxin administration (Table 2). Thus, it appears that endothelial activation by PARs may be unnecessary for sequestration of neutrophils in the microvasculature in the setting of endotoxemia.

Rather than conferring protection to endotoxin, PAR deficiency appeared to worsen survival in at least one case. Decreased survival of Par1−/− mice was a relatively consistent finding (Figure 3). In the context of decreased survival noted in mice with genetic (Nf-E2−/−) or immune thrombocytopenia (Figure 7), this result suggests that platelet function plays a net protective role in this model of endotoxemia. Whether platelets are simply effecting hemostasis and/or mitigating the effects of endothelial damage and whether treating severe thrombocytopenia would provide benefit in this model are unknown.

We interpret the apparent decreased survival of Par1−/−: Par2−/− mice in this study (Figure 5) with extreme caution, because embryonic lethality has precluded our performing this study in an inbred background or with proper littermate controls. It is certainly possible that the difference between Par1−/−: Par2−/− mice and the controls seen at low-dose endotoxin (Figure 5) was due not to loss of PAR function but to differences in genetic background selected for by their 5% survival rate. Moreover, combining hirudin treatment with PAR2 deficiency yielded neither decreased nor increased survival, but it is possible that the level of thrombin inhibition achieved in this study was not sufficient to
mimic PAR1 deficiency or that hirudin had both positive and negative effects. Regardless, the apparent decreased survival of Par1<sup>-/-</sup>/Par2<sup>-/-</sup> mice at low-dose endotoxin is certainly consistent with our general finding that PAR1 and PAR2 activation does not play an important role in promoting inflammation or decreasing survival in this model. Decreased survival of Par1<sup>-/-</sup>/Par2<sup>-/-</sup> mice is also interesting in the context of potential antiapoptotic and cytoprotective effects of endothelial PARs and is at least consistent with the possibility that PAR1 and PAR2 might play an endothelial-protective role in settings of DIC and systemic inflammation. Recent in vitro evidence that APC and perhaps low-level thrombin signaling through PAR1<sup>18</sup> and Xa signaling through PAR1 and PAR2<sup>23</sup> can protect endothelial barrier integrity is also consistent with this notion.

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**References**

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Eric Camerer, Ivo Cornelissen, Hiroshi Kataoka, Daniel N. Duong, Yao-Wu Zheng and Shaun R. Coughlin

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