

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

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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 exam-

ined 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 antithrombin 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.¹ 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,²⁻⁴ and endothelial damage from leukocyte products can allow circulating coagulation factors access to extravascular TF and impair natural anticoagulant pathways.^{1,5,6} 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.^{1,7,8}

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.⁹⁻¹⁶ In cell culture, proinflammatory as well as cytoprotective cellular responses to coagulation factors have been described.^{9,17-19} 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,²⁰⁻²³ while other inhibitors (Xai) prevent DIC but do not affect cytokine responses or survival.²⁴ In a mouse model of *E coli* sepsis, inhibition of TF/VIIa did not affect inflammation,²⁵ but mice with low TF expression did show a

decreased cytokine response to endotoxin.³ Activated protein C (APC), an endogenous anticoagulant that is activated by thrombin, is protective in sepsis,^{26,27} but this effect may be due to activities of APC that are unrelated to its anticoagulant function.^{17,18,28,29} Thus, inhibition of coagulation might decrease antiinflammatory effects of thrombin, and how this balance might play out in various settings is unknown. Better characterization of the mechanisms by which coagulation might modulate inflammation in endotoxemia and a better sense of their relative importance are needed.

Protease-activated receptors (PARs) connect coagulation proteases to cellular responses and represent one mechanism by which coagulation might affect inflammation.³⁰ The literature surrounding the potential roles of these receptors in systemic responses to endotoxin is active and controversial.^{31,32} Of the 4 mammalian PARs, PAR1, PAR3, and PAR4 are activated by thrombin,³³⁻³⁵ and PAR2 can be activated by coagulation proteases VIIa and Xa but not thrombin.^{36,37} In mouse, PAR4 is necessary for platelet activation by thrombin³⁸; PAR1 is the main thrombin receptor on microvascular endothelial cells and mesenchymal cells,^{39,40} and PAR1 and PAR2 together account for TF/VIIa and Xa signaling in these cells.⁴¹ PAR1 is necessary for thrombin responses in smooth muscle cells and fibroblasts.^{39,42,43} 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.

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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*, *F2r11*, and *F2r13*, respectively. Generation of *Par1*^{-/-}, *Par2*^{-/-}, and *Par4*^{-/-} mice has been described.^{38,39,44,45} *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*^{-/-}) mice⁴⁵ and Nf-E2-deficient (*Nf-E2*^{-/-}) mice⁴⁶ were generously provided by Drs Jay L. Degen (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 C57BL/6 background, *Par1*^{-/-}:*Par2*^{-/-}, *Par2*^{-/-} (littermate study), *Fib*^{-/-}, and *Nf-E2*^{-/-} lines were maintained in a 129/SVJ:C57BL/6 mixed background (referred to as 129/B6). Initial studies of *Par1*^{-/-}, *Par2*^{-/-}, and *Par4*^{-/-} mice were done in the C57BL/6 background and compared with mice generated by homozygous (-/-) 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 monitoring were considered moribund and were immediately killed. These animals were included in the survival curves and accounted for approximately 20% of all deaths. Recombinant hirudin (Refludan; kindly provided by Berlex Biosciences, Richmond, CA) or saline control was injected intraperitoneally 30 minutes before and 30 minutes, 2 hours, and 4 hours after endotoxin at indicated concentrations as described by Pawlinski et al.³ For cytokine measurements at 8 hours, an additional injection was done at 6 hours. The synthetic S1P1 agonist precursor FTY720 (0.1 mg/kg; kindly provided by Dr Jason Cyster, University of California at San Francisco) or saline control was injected intraperitoneally 30 minutes prior to endotoxin. Antiplatelet serum (100 μ L 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 μ L 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 anemia 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 $300 \times 10^9/L$ ($300 \times 10^3/\mu L$) to $1000 \times 10^9/L$ ($1000 \times 10^3/\mu L$) but diverged when counts were below $200 \times 10^9/L$ ($200\,000/\mu L$), 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 TNF α and IL-1 β ,^{3,23} 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 μ Ci/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 myoinositol 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 μ m 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 \times 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 LD_{50} 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 $970 \times 10^9/L$ to $480 \times 10^9/L$, $310 \times 10^9/L$, and $270 \times 10^9/L$ 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

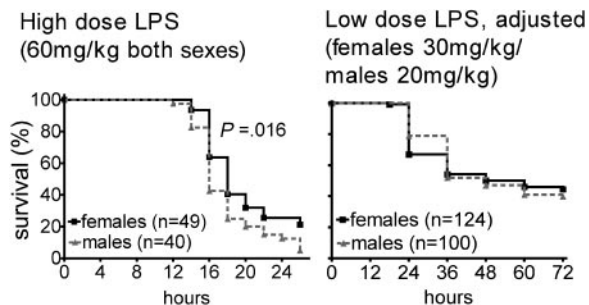


Figure 1. Sex differences in survival after endotoxin. N5 C57BL/6 females (solid lines) and males (dotted lines) from the colonies used for the studies in Figures 2-6 were injected intraperitoneally with high-dose LPS (60 mg/kg; left) or low-dose LPS (30 mg/kg for females, 20 mg/kg for males; right) and monitored for 26 and 72 hours, respectively. *P* values as determined by log-rank test are shown only when less than .05.

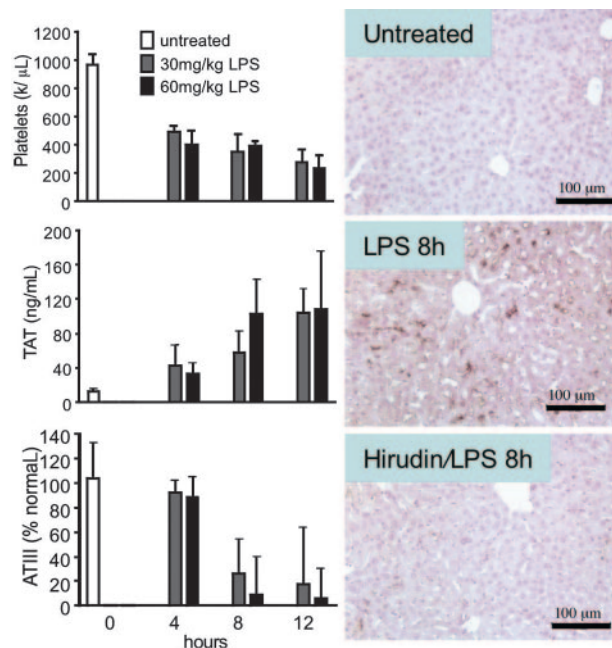


Figure 2. Characterization of disseminated intravascular coagulation after endotoxin. (Left) C57BL/6 females were injected intraperitoneally with 30 (low dose) or 60 (high dose) mg/kg LPS. At the indicated time, blood was collected and platelet counts, TAT levels, and ATIII levels determined (mean \pm SD, $n = 6$). (Right) C57BL/6 females were injected intraperitoneally with 60 mg/kg LPS with or without hirudin (5 mg/kg) treatment as described in “Materials and methods”; untreated control mice were processed in parallel. At 8 hours, mice were perfusion fixed and tissues processed for immunohistochemistry. Antifibrin immunostaining of paraffin sections of liver is shown. Note fibrin deposition in hepatic microvessels of LPS-treated mice and its prevention by hirudin.

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

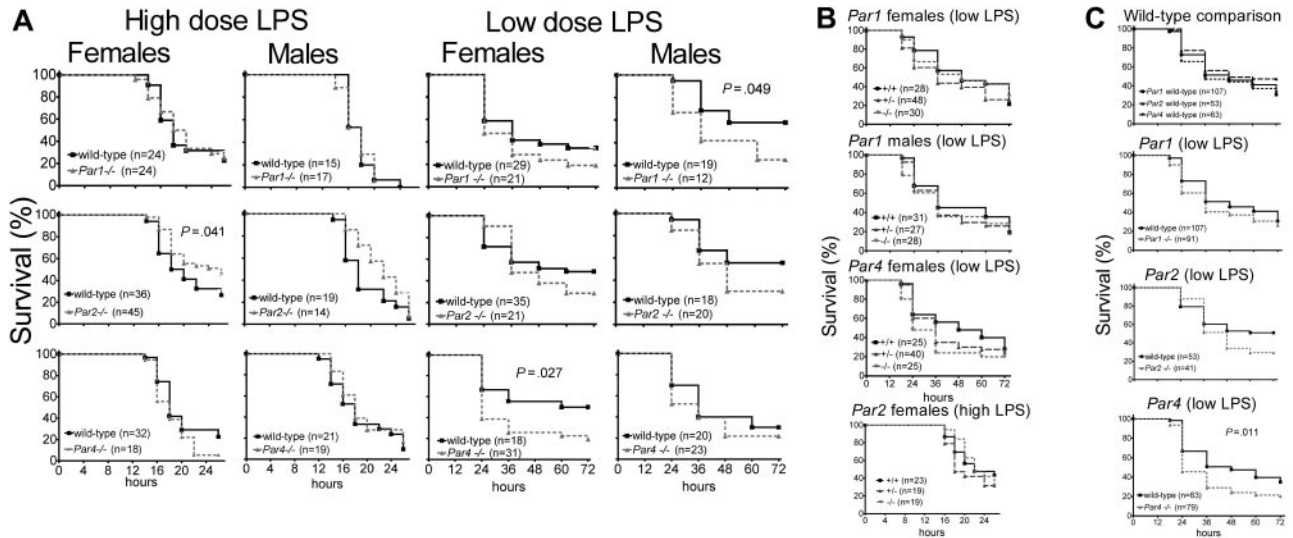


Figure 3. Survival of PAR-deficient mice after endotoxin challenge: single knockouts. (A) *Par1*^{-/-} (top panels), *Par2*^{-/-} (middle panels), and *Par4*^{-/-} (bottom panels) mice and strain- (about 97.5% C57BL/6, about 2.5% 129SVJae), age-, and sex-matched controls were injected intraperitoneally 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 responses. 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.

results. Toward addressing these issues, we repeated the 4 comparisons that showed interesting trends in Figure 3A (PAR1 males and females at low-dose endotoxin, PAR4 females at low dose, and PAR2 females high dose), this time using littermate controls from heterozygote crosses. No significant differences between groups were detected (Figure 3B), but a trend toward increased sensitivity to endotoxin was again noted in the *Par4*^{-/-} low-dose group. An analysis of pooled data from experiments of similar design from Figure 3A-B is shown in Figure 3C. *Par4*^{-/-} mice showed decreased survival after low-dose endotoxin in this pooled analysis, consistent with the trend seen in the individual studies. Survival of *Par1*^{-/-} and *Par2*^{-/-} groups was not significantly different from controls, and the trend, if any, was for decreased rather than increased survival in PAR-deficient mice. Thus, we detected no role for PAR1, PAR2, or PAR4 in promoting death from endotoxemia using isolated PAR deficiencies under the conditions studied. We of course cannot exclude the possibility that such roles might be detected if still other conditions, such as lower dose endotoxin and lower mortality models, were explored.

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 anrod treatment was shown to be protective in the same study.³ 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

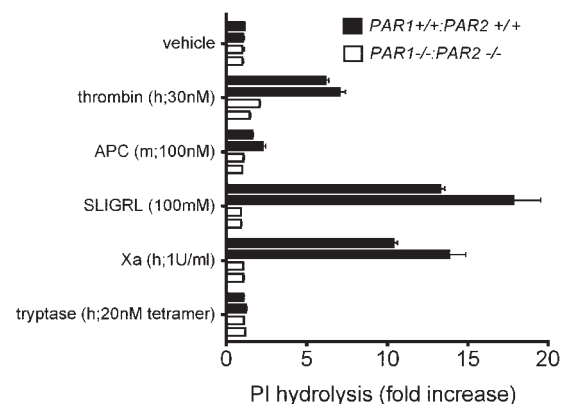


Figure 4. Protease signaling in endothelial cells from *Par1*^{-/-}:*Par2*^{-/-} mice. Dermal microvascular endothelial cells isolated from *Par1*^{-/-}:*Par2*^{-/-} neonates or matched controls were serum-starved, loaded with ³H-inositol, and exposed to vehicle, protease, or agonist peptide as indicated. To exclude nonspecific responses to contaminating thrombin, hirudin was included with all proteases except thrombin. Results from 2 independent experiments are shown. Each bar represents 1 experiment done in triplicate; error bars show standard deviation. *Par1*^{-/-}:*Par2*^{-/-} cells did respond to nonprotease agonists such as serum and lysophosphatidic acid; h indicates human; m, mouse.

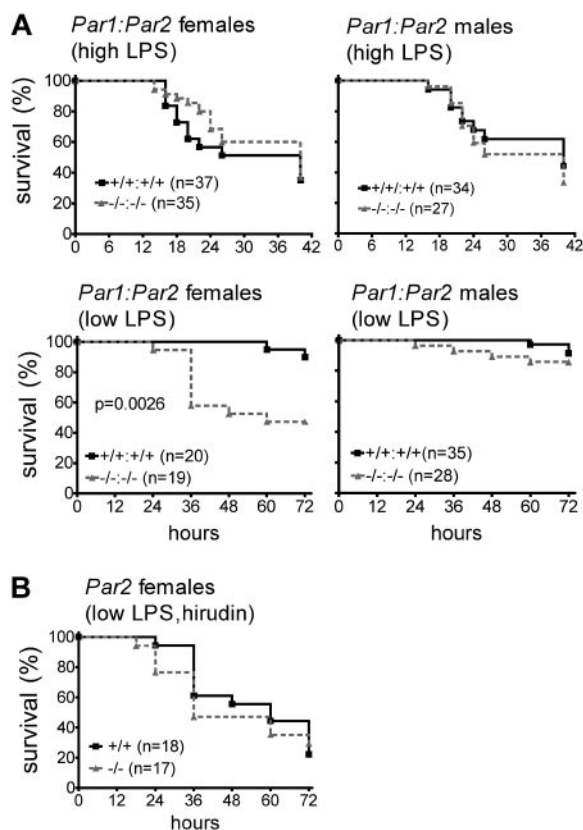


Figure 5. Survival of PAR-deficient mice after endotoxin challenge: double knockouts. (A) *Par1*^{-/-}:*Par2*^{-/-} (50% C57BL/6, 50% 129SVJae) mice and strain-, age-, and sex-matched controls were injected intraperitoneally with LPS and survival monitored as indicated. (B) *Par2*^{-/-} (about 97.5% C57BL/6, about 2.5% 129SVJae) females and littermate controls were injected intraperitoneally with hirudin (1 mg/kg) 30 minutes before and 30 minutes, 2 hours, and 4 hours after intraperitoneal injection of low-dose LPS.

that Xa signals mainly through PAR1 and PAR2⁴¹ and supporting the idea that PARs account for signaling responses to APC in cultured endothelial cells.^{18,49} Trypsase and proteinase 3, both leukocyte granzymes and candidate PAR-activating proteases⁵³⁻⁵⁶ that might be present in endotoxemia, did not elicit responses even in wild-type controls (Figure 4; not shown). Based on these in vitro findings, we used *Par1*^{-/-}:*Par2*^{-/-} mice to probe the importance of endothelial cell responses to coagulation proteases in endotoxemia.

Even in the mixed 129Sv/C57BL6 background, *Par1*^{-/-}:*Par2*^{-/-} mice were born at about 5% of the expected mendelian rate, but the surviving mice were fertile. Thus, for practical reasons, *Par1*^{-/-}:*Par2*^{-/-} mice were generated from het-null and null-null crosses, and age- and sex-matched wild-type controls from the same colony were used. Compared with such controls, *Par1*^{-/-}:*Par2*^{-/-} mice showed no difference in survival or cytokine levels in response to high-dose endotoxin (Figure 5A; Table 1). However, *Par1*^{-/-}:*Par2*^{-/-} female mice appeared to have decreased survival and increased cytokine production to low-dose endotoxin, and a similar trend was noted in male mice (Figure 5A; Table 1). The overall mortality in this experiment was decreased compared with that seen in our studies in the C57BL6 background, probably due to the partial 129Sv background and emphasizing the possibility that chance differences in the strain mixture might influence outcome.⁵⁷ Indeed, while the matched controls used in these studies were of the same average strain composition as the knockouts (50:50 129SvJae/C57BL6), we cannot exclude the possibility that survival of

Par1^{-/-}:*Par2*^{-/-} mice selected for particular 129SvJae or C57BL6 loci, nor can we exclude the possibility that more random variation in the contribution from the 129SvJae and C57BL6 genomes influenced outcome.

Toward examining the effect of knocking down PAR1 and PAR2 function with a better-matched control group, we were able to generate sufficient *Par2*^{-/-} and *Par2*^{+/+} littermates in a C57BL6 background to attempt to unmask a PAR2-dependent change in the response to endotoxin by use of the thrombin inhibitor hirudin.³ Despite inhibiting TAT formation and microvascular fibrin deposition, hirudin treatment failed to reveal either sensitization or protection of *Par2*^{-/-} mice (Figure 5B; Tables 1-2).

PAR2 and PAR4 (but not PAR1) agonists have been reported to be sufficient to trigger local leukocyte margination in mouse models,^{58,59} but cytokine responses in *Par2*^{-/-}:*Par4*^{-/-} mice were not different from wild type, and these mice showed a trend toward decreased, not increased, survival (Tables 1-2; not shown).

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) or 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 mice³⁸ (Figure 6). In accord with equally decreased platelet counts, platelet accumulation in the liver microvasculature appeared similar in *Par4*^{-/-} mice and their 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 $307 \times 10^9/L \pm 92 \times 10^9/L$ in *Par4*^{-/-} versus $233 \times 10^9/L \pm 98 \times 10^9/L$ 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.

Table 1. TAT and cytokine responses 8 hours after LPS

Treatments and genotype by sex and strain	No. of mice	TAT, ng/mL (SD)	IL-6, ng/mL (SD)	MIP-2, ng/mL (SD)	MCP-1, ng/mL (SD)	IL-10, pg/mL (SD)
F, C57BL/6						
Thrombin inhibition, high-dose LPS						
wt, saline/saline	8	1.6 (0.96)	0 (7.6)	0 (3.4)	0.27 (0.38)	13.7 (5.9)
wt, saline/LPS	8	78.5 (43.1)	406 (325)	151 (92.5)	568 (551)	793 (432)
wt, 5 mg/kg hirudin/LPS	7	0.78 (0.37)*	484 (333)	199 (82.5)	729 (589)	1109 (458)
Thrombin inhibition, low-dose LPS						
wt, saline/LPS	8	48.7 (30.0)	142 (84.4)	94.2 (68.8)	205 (120)	781 (318)
wt, 1 mg/kg hirudin/LPS	8	14.2 (3.96)*	65.9 (27.7)	39.5 (19.6)	104 (32.9)	865 (284)
wt, 5 mg/kg hirudin/LPS	6	4.29 (0.49)*	88.7 (61.5)	50.6 (40.0)	112 (45.8)	1188 (1072)
Par2/Par4 low-dose LPS						
wt: no pretreatment/LPS	6	80.6 (32.0)	57.7 (48.7)	31.3 (29.4)	167 (96.2)	1101 (579)
ko: no pretreatment/LPS	7	91.7 (52.9)	44.5 (11.7)	36.9 (23.6)	133 (54.3)	932 (423)
Thrombocytopenia low-dose LPS						
wt, control serum/LPS	8	64.8 (19.0)	67.5 (36.7)	47.2 (32.9)	113 (58.4)	905 (491)
wt, antiplatelet serum/LPS	8	72.6 (13.9)	110 (40.5)	53.8 (19.7)	139 (29.9)	1464 (731)
M, 129/C57BL/6						
Thrombin inhibition PAR2 littermates, low-dose LPS						
wt, 1 mg/kg hirudin/LPS	11	2.8 (1.5)	34.2 (29.5)	ND	ND	ND
ko, 1 mg/kg hirudin/LPS	11	2.7 (1.3)	29.2 (28.8)	ND	ND	ND
Par1/Par2 high-dose LPS						
wt, no pretreatment/LPS	6	22.5 (3.68)	23.4 (10.5)	17.7 (6.44)	64.0 (18.0)	816 (307)
ko, no pretreatment/LPS	7	137 (41.0)†	69.3 (95.8)	64.8 (63.5)	137 (136)	1811 (2057)
Par1/Par2 low-dose LPS						
wt, no pretreatment/LPS	5	131 (91.5)	28.4 (22.2)	3.83 (2.03)	43.8 (31.9)	305 (117)
ko, no pretreatment/LPS	5	60.3 (25.5)	43.7 (27.0)	11.6§ (5.52)	83.0 (40.0)	443 (214)
Fibrin(ogen) low-dose LPS						
wt, no pretreatment/LPS	5	29.4 (6.92)	35.4 (47.7)	42.3 (69.9)	118 (165)	575 (313)
ko, no pretreatment/LPS	5	37.5 (17.3)	43.2 (46.8)	23.9 (17.4)	67.3 (45.3)	442 (214)

F Indicates female; M, male; ND, not determined.

* $P < .01$, relative to the LPS-treated control group.† $P < .05$, relative to the LPS-treated control group.

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 antiplatelet 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 reported to prevent the barrier-disruptive effect of thrombin and VEGF in endothelial cultures.^{18,64} However, administration of FTY720, an S1P1 agonist, at a dose known 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

Table 2. Blood cell counts 8 hours after LPS

Treatment and genotype by sex and strain	No. of mice	Lymphocyte count, $\times 10^9/L$ (SD)	Neutrophil count, $\times 10^9/L$ (SD)	Monocyte count, $\times 10^9/L$ (SD)	RBC count, $\times 10^{12}/L$ (SD)	Platelet count, $\times 10^9/L$ (SD)
F, C57BL/6						
Thrombin inhibition, high-dose LPS						
wt, saline/saline	8	ND	ND	ND	ND	988 (89)†
wt, saline/LPS	8	ND	ND	ND	ND	283 (85)†
wt, 5 mg/kg hirudin/LPS	7	ND	ND	ND	ND	340 (88)†
Thrombin inhibition, low-dose LPS						
wt, saline/LPS	8	1.8 (0.7)	0.30 (0.14)	0.12 (0.04)	9.7 (0.7)	393 (65)
wt, 1 mg/kg hirudin/LPS	8	1.4 (0.5)	0.33 (0.12)	0.12 (0.03)	9.7 (0.9)	358 (102)
wt, 5 mg/kg hirudin/LPS	6	2.1 (1.0)	0.26 (0.17)	0.17 (0.09)	7.5 (1.7)‡	355 (68)
Par2/Par4 low-dose LPS*						
wt: no pretreatment/LPS	6	1.2 (0.2)	0.15 (0.07)	0.05 (0.02)	9.0 (1.0)	469 (16)
ko: no pretreatment/LPS	7	0.92 (0.18)	0.10 (0.02)	0.04 (0.02)	8.5 (0.4)	537 (68)
Thrombocytopenia low-dose LPS						
wt, control serum/LPS	8	2.0 (0.4)	0.46 (0.12)	0.14 (0.03)	9.4 (0.5)	401 (68)
wt, antiplatelet serum/LPS	8	1.6 (0.6)	0.28 (0.13)§	0.08 (0.03)‡	8.4 (0.7)	187 (95)‡
M, 129/C57BL/6						
Thrombin inhibition PAR2 littermates, low-dose LPS						
wt, 1 mg/kg hirudin/LPS	11	1.5 (0.8)	0.39 (0.28)	0.22 (0.09)	9.5 (0.7)	403 (95)
ko, 1 mg/kg hirudin/LPS	11	1.8 (0.6)	0.51 (0.24)	0.29 (0.14)	9.6 (0.3)	358 (88)
Par1/Par2 high-dose LPS*						
wt, no pretreatment/LPS	6	1.1 (0.4)	0.10 (0.08)	0.08 (0.04)	9.3 (0.6)	432 (49)
ko, no pretreatment/LPS	7	1.3 (0.5)	0.30 (0.22)	0.12 (0.08)	9.7 (0.8)	505 (193)
Par1/Par2 low-dose LPS						
wt, no pretreatment/LPS	5	2.8 (1.4)	0.79 (0.48)	0.37 (0.14)	10.7 (1.3)	394 (100)
ko, no pretreatment/LPS	5	1.3 (0.5)§	0.41 (0.12)	0.15 (0.06)§	11.2 (0.7)	489 (71)
Fibrin(ogen) low-dose LPS						
wt, no pretreatment/LPS	5	2.6 (1.2)	0.80 (0.61)	0.36 (0.25)	8.3 (0.7)	294 (61)
ko, no pretreatment/LPS	5	2.4 (1.7)	0.45 (0.25)	0.21 (0.01)	8.8 (1.4)	305 (36)

RBC indicates red blood cell; F, female; M, male; ND, not determined.

*CBC measurements 3 hours after LPS.

†Platelet counts determined by manual counts.

‡ $P < .01$, relative to LPS-treated control group.

§ $P < .05$, relative to LPS-treated control group.

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 endotoxemia model as was combined PAR2 deficiency and throm-

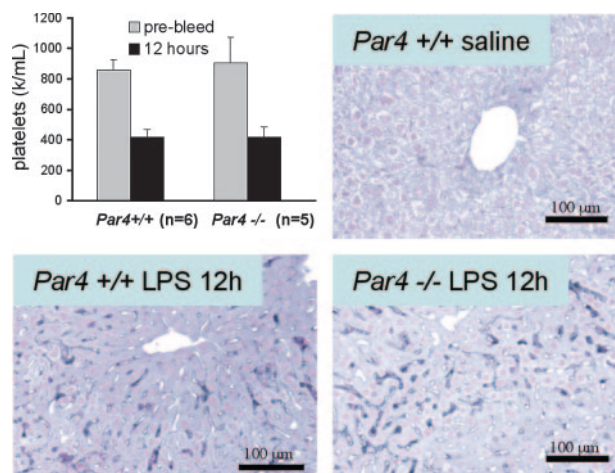


Figure 6. Endotoxin-induced thrombocytopenia in *Par4*^{-/-} mice. *Par4*^{-/-} males or littermate control males were injected intraperitoneally with 20 mg/kg LPS. Twelve hours later, mice were bled for platelet counts and perfused for immunohistochemistry. (Top left, mean \pm SD) Platelet counts in *Par4*^{-/-} and littermate controls before and after LPS treatment. (Top right and bottom) Antiplatelet immunostaining of paraffin sections of liver from an LPS-treated *Par4*^{-/-} or *Par4*^{+/+} mouse or a saline-treated *Par4*^{+/+} control. Note immunostaining for platelets in microvessels of LPS-treated mice regardless of genotype.

bin inhibition.³ However, others have reported that inhibition of TF/VIIa failed to protect against inflammation or death in a mouse model of *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 inflammation in the setting of endotoxemia; nor do they imply that PARs do not contribute importantly to other types of 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 platelet aggregation and sequestration in the microvasculature. Platelets from *Par4*^{-/-} mice are unresponsive to even micromolar concentrations of thrombin,³⁸ and the persistence of endotoxin-induced platelet accumulation in the microvasculature and thrombocytopenia in *Par4*^{-/-} mice (Figure 6) suggests that platelet activation by thrombin is not necessary for thrombocytopenia in

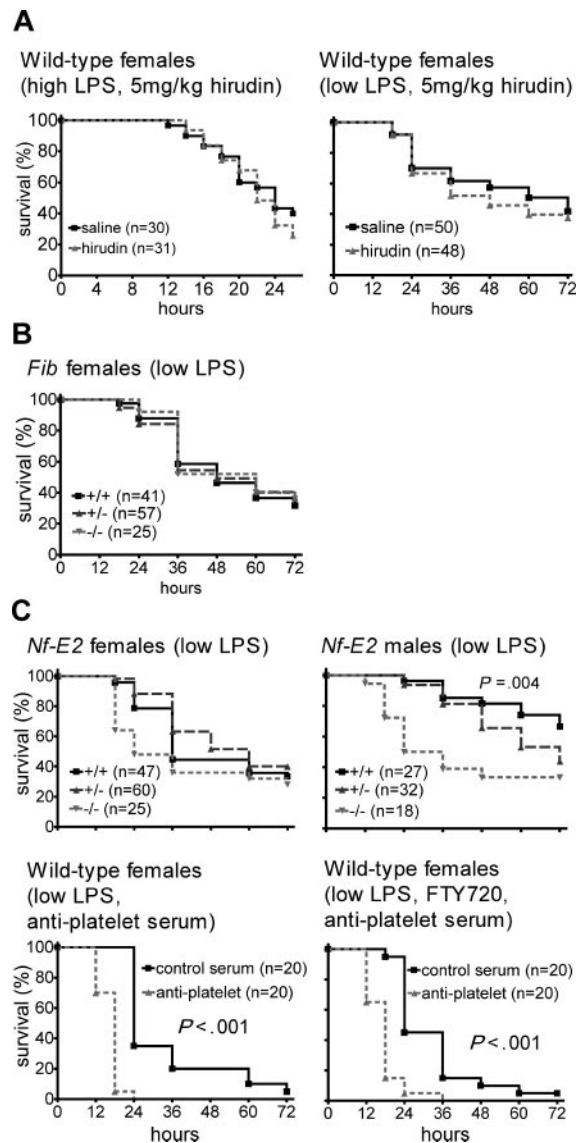


Figure 7. Effect of inhibition of thrombin, fibrin(ogen), or platelet function upon endotoxin mortality. Effects of thrombin inhibition (A) or lack of fibrinogen (B) or platelets (C) on endotoxin mortality. Wild-type C57BL/6 female mice treated with hirudin (5 mg/kg intraperitoneally), antiplatelet serum, and/or FTY720 (0.1 mg/kg intraperitoneally) and *Fib*^{-/-} or *Nf-E2*^{-/-} (both 50% C57BL/6, 50% 129SVJ) mice and appropriate vehicle- or nonimmune serum-injected or littermate controls were injected intraperitoneally with high- or low-dose LPS and monitored for 26 or 72 hours as indicated. *P* values as determined by log-rank test are shown when less than .05. For littermate studies, *P* value refers to comparison between knockouts and wild types.

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 $100 \times 10^9/L$) 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*^{-/-} 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.⁷⁵ The endotoxin receptor TLR4 is expressed on platelets, and endotoxin can promote platelet adhesion to fibrinogen in a flow chamber assay.⁷⁵ 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.⁷⁵ 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.⁷⁶ This system would also be eliminated in the *Par4* knockout (if it even operates in mice).^{38,76} 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^{59,77}; 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)⁷⁸; and subcutaneous injection of PAR agonists triggers edema and neutrophil infiltration.⁷⁹⁻⁸¹ 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 *Par4*^{-/-} 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*^{-/-}:*Par2*^{-/-} 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*^{-/-}:*Par2*^{-/-} mice is also interesting in the context of potential antiapoptotic and cytoprotective effects of endothelial PARs²⁹ 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 PAR1¹⁸ and Xa signaling through PAR1 and PAR2⁸² can protect endothelial barrier integrity is also consistent with this notion.

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