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*J Immunol* 2011; 187:1866-1876; Prepublished online 1 July 2011;
doi: 10.4049/jimmunol.1101094
http://www.jimmunol.org/content/187/4/1866
Protective Roles for Fibrin, Tissue Factor, Plasminogen Activator Inhibitor-1, and Thrombin Activable Fibrinolysis Inhibitor, but Not Factor XI, during Defense against the Gram-Negative Bacterium Yersinia enterocolitica

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Septic infections dysregulate hemostatic pathways, prompting coagulopathy. Nevertheless, anticoagulant therapies typically fail to protect humans from septic pathology. The data reported in this work may help to explain this discrepancy by demonstrating critical protective roles for coagulation leading to fibrin deposition during host defense against the Gram-negative bacterium Yersinia enterocolitica. After i.p. inoculation with Y. enterocolitica, fibrinogen-deficient mice display impaired cytokine and chemokine production in the peritoneal cavity and suppressed neutrophil recruitment. Moreover, both gene-targeted fibrinogen-deficient mice and wild-type mice treated with the anticoagulant coumadin display increased hepatic bacterial burden and mortality following either i.p. or i.v. inoculation with Y. enterocolitica. Mice with low tissue factor activity succumb to yersiniosis with a phenotype similar to fibrin(ogen)-deficient mice, whereas factor XI-deficient mice show wild-type levels of resistance. Mice deficient in plasminogen activator inhibitor-1 or thrombin-activatable fibrinolysis inhibitor display modest phenotypes, but mice deficient in both plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor succumb to yersiniosis with a phenotype resembling fibrin(ogen)-deficient mice. These findings demonstrate critical protective roles for the tissue factor-dependent extrinsic coagulation pathway during host defense against bacteria and caution that therapeutics targeting major thrombin-generating or antifibrinolytic pathways may disrupt fibrin-mediated host defense during Gram-negative sepsis. The Journal of Immunology, 2011, 187: 1866–1876.

Hemostatic blood coagulation pathways culminate in the deposition of fibrin, a provisional extracellular matrix that contributes to blood clotting and wound repair. Fibrin levels are regulated by fibrin-promoting coagulant pathways and fibrin-degrading fibrinolytic pathways. During sepsis, a major cause of death in hospitals, hemostasis becomes unbalanced: up-regulated procoagulant activity is insufficiently balanced by anti-coagulation and fibrinolysis, prompting disseminated intravascular coagulation (DIC) and fibrin deposition (1, 2). In addition to promoting ischemia and inflammation, DIC eventually consumes critical blood-clotting factors, thus contributing to hemorrhage and shock. This constellation of coagulopathies is difficult to treat and contributes to the high mortality rates for patients presenting with sepsis (1, 2).

Whereas DIC contributes to septic pathology, coagulation leading to fibrin deposition also can perform critical protective functions during infection. Mice lacking the capacity to produce fibrin (e.g., gene-targeted fibrinogen-deficient mice or coumadin-treated wild-type mice) succumb prematurely to infection with Toxoplasma gondii (3). The premature deaths appear to result from hemorrhage caused by the immune system as it clears this protozoan parasite from the liver. Likewise, fibrin deficiency prompts acute hepatic hemorrhage 5 d after inoculation of mice with Listeria monocytogenes, a Gram-positive bacterium (4). Thus, fibrin-mediated hemostatic immunity can be critical to surviving certain protozoan and bacterial infections.

In addition to restraining hemorrhage, fibrinogen also functions protectively by limiting the growth and/or dissemination of certain pathogens. After inoculation with the Gram-positive bacteria L. monocytogenes or Staphylococcus aureus (4, 5), but not after inoculation with T. gondii (3), fibrinogen-deficient mice exhibit greatly increased pathogen burden. One potential explanation is that physical entrapment of bacteria by fibrin may limit their capacity to disseminate (6, 7). Additionally, fibrinogen may facilitate bacterial clearance by phagocytes: fibrinogen is a ligand for CD11b/CD18 and CD11c/CD18 integrins expressed by leukocytes (8, 9), and studies of gene-targeted fibrinogen-mutant mice suggest that fibrinogen stimulates inflammation, leading to the recruitment and activation of leukocytes (5, 10, 11). Many bacteria produce factors that bind to fibrin and/or regulate fibrin levels, presumably as a means to counter fibrin-mediated host defense mechanisms (12, 13).

Given the protective and pathological potential of fibrin during infection, the development of therapeutics that safely suppress septic coagulopathy while maintaining protective hemostasis and...
other critical elements of fibrin-dependent host defense may require a thorough understanding of hemostatic pathway regulation during infection. Already, a great deal is known about the regulation of hemostasis during vascular trauma and thrombosis. Procoagulant pathways initiate fibrin formation by stimulating production of thrombin, a protease that cleaves soluble fibrinogen, prompting its polymerization and deposition as insoluble fibrin. Tissue factor (TF) plays a prominent role in the initiation of vascular procoagulant pathways (14). TF is expressed primarily by extravascular cells, whereas the proteases that generate thrombin circulate in plasma as inactive precursors. This physical segregation usually ensures that this extrinsic coagulation pathway is only activated in response to breaches of vascular integrity. However, procoagulant pathways also may be activated by inflammation-induced upregulation of TF on cells within the vasculature (2, 14), or by TF-independent pathways, such as the factor XI (FXI)-dependent intrinsic coagulation pathway (12, 15). Regardless of the initiating mechanisms, procoagulant pathways all culminate in the formation of a prothrombinase (PT) complex that produces thrombin, the protease that cleaves fibrinogen, thereby prompting its polymerization and deposition as fibrin. These procoagulant activities are limited by multiple anticoagulant mechanisms, including TF pathway inhibitor, antithrombin, and activated protein C (2, 16).

Once formed, fibrin levels are regulated by plasmin, a fibrin-degrading protease derived from plasminogen upon its proteolytic activation by plasminogen activators (PA). Fibrinolysis is negatively regulated by multiple factors, including PA inhibitor 1 (PAI-1) (17), an inflammation-inducible PA antagonist whose levels increase during sepsis (18), and thrombin activatable fibrinolysis inhibitor (TAFI), an enzyme that indirectly suppresses plasminogen activation by modifying fibrin in a manner that reduces its affinity for plasminogen and PA (19, 20).

Therapeutic targeting of TF and other elements of the extrinsic coagulation pathway can lessen pathology and improve survival in animal models of bacterial sepsis (2, 14, 21, 22). However, many human clinical trials for sepsis have failed to demonstrate that treatment with anticoagulants can significantly improve survival (1, 2). Only one anticoagulant, recombinant human activated protein C, has been licensed for the treatment of severe sepsis (16, 23), and the overall therapeutic benefit of recombinant human activated protein C has been questioned, in part because serious bleeding is a significant complication in patients treated with this potent anticoagulant (24). Therapeutic strategies based on partial anticoagulation, for example, via depletion of FXI (25), or based on augmentation of fibrinolysis, for example, by antagonizing PAI-1 and/or TAFI (18, 26, 27), are under investigation.

Gram-negative bacteria are a common cause of sepsis and sepsis-associated coagulopathy (1, 28). Animal models of sepsis commonly employ bolus injections of the Gram-negative bacterium Escherichia coli or its endotoxin as a challenge. Typically, these models lead to intoxication, rather than colonization and sustained infection (29–31). Host defense roles for coagulation and fibrin deposition may be dispensable in intoxication models of Gram-negative sepsis, but critical in settings of sustained infection.

Yersinia enterocolitica is a Gram-negative bacterium that establishes sustained infections in mice that must be cleared by a robust host response (32, 33). Transmission of Y. enterocolitica to humans typically follows the ingestion of contaminated food, water, and milk (34). The resulting yersiniosis typically manifests as a self-limiting enterocolitis; however, Y. enterocolitica also can cause extraintestinal disorders, including sepsis (34, 35). Moreover, transfusion-associated septic yersiniosis, which results from the growth of Y. enterocolitica in refrigerated blood, has >35% case fatality rates (34, 36–38). In this study, we demonstrate that fibrin performs multiple protective functions in the C57BL/6 mouse model of yersiniosis, including the induction of cytokine and chemokine expression, activation of neutrophil recruitment, and restraint of bacterial burden. Investigations of the mechanisms regulating the deposition of protective fibrin revealed critical roles for TF, but not FXI. Our studies also revealed synergy in the antifibrinolytic functions of PAI-1 and TAFI. We discuss the relevance of these findings to the development of sepsis therapeutics that aim to limit coagulopathy without preventing the deposition of protective fibrin.

Materials and Methods

Mice

C57BL/6 wild-type and PAI-1–deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 fibrinogen-deficient mice (39), FXI-deficient mice (40), and TAFI-deficient mice (41) were supplied by J. Degen (Cincinnati Children’s Hospital Medical Center), D. Galiani, and E. Plow, respectively. PAI-1/TAFI-deficient mice were generated at Trudeau Institute. N. Mackman supplied C57BL/6 mice with very low levels of TF activity (low-TF mice). These mice lack expression of mouse TF due to its inactivation by gene targeting and instead express a human TF transgene, which imparts low-level TF activity (mTF−/− hTF+) (42). Low-TF mice were compared with littermates heterozygous for mouse TF and expressing human TF (mTF+/− hTF+). When littermate fibrinogen-deficient mice, FXI-deficient mice, or TF transgenic mice were compared, they were cohoused with controls and the investigators were blinded with regard to the animals’ genotypes. All experimental mice were bred in a specific pathogen-free facility at Trudeau Institute. They were matched for age and sex, and infected between 6 and 10 wk of age. Where indicated, mice were anticoagulated pharmacologically by supplementing drinking water with 2 mg/l coumadin [3-([α-acetonylbenzyl]-4-hydroxycoumarin; Sigma-Aldrich] beginning 3 d prior to infection or 1 d post-infection with replenishment every 48 h; this anticoagulant regimen reduces fibrin deposition in mice during infection (4). All animal studies were conducted in accordance with Trudeau Institute Animal Care and Use Committee guidelines.

Bacterial infection

Y. enterocolitica strain WA (serotype O:8) (32) was obtained from American Type Culture Collection (27729). After growth to early log phase at 26°C in brain heart infusion broth (BHI; Difco Laboratories, Detroit, MI), bacteria were resuspended in BHI supplemented with 20% glycerol and stocks were stored as single-use aliquots at −70°C. Preliminary studies established that the i.v. dose that caused half the mice to succumb to infection (LD50) was 5 × 105 CFU. For experimental infections, frozen bacteria were diluted with saline to the desired dose and administered in a 100 μl vol. The number of bacteria in the inoculating dose was confirmed by plating on BHI agar.

Measurements of survival and bacterial burden

Mice were monitored daily. Unresponsive or recurrent animals were considered moribund and euthanized. For measurement of bacterial burden, fluids and tissues were collected after mice were euthanized by carbon dioxide narcosis. Bacterial burden was measured by homogenizing tissues in saline, plating serial dilutions on BHI agar, incubating overnight at 26°C, and counting CFU.

Measurements of blood parameters, fibrin deposition, and hepatic PT and PA activity

Blood was collected by cardiac puncture from mice that received 500 U heparin i.v. just prior to euthanasia. After dilution in saline containing 5 mM EDTA, hematocrits and platelet numbers were measured using a Coulter counter (Beckman). Plasma levels of D-dimer were measured by ELISA (Diagnostica Stago) and reported as fold-change relative to the average value measured in naive C57BL/6 mice. Levels of fibrin in liver tissue were quantified as described (3) and scored positive when above the limit of detection (15 ng/mg tissue). In brief, fibrin was extracted from homogenized liver tissue and quantified by Western blot using fibrin-specific mAb 3B5 (American Diagnostic); the standard curve was prepared from mouse fibrinogen treated with thrombin (3). Levels of PT and PA activity in liver tissue were quantified in situ using validated assays (43). In brief, fresh-frozen livers were sectioned (7 μm), mounted onto slides, and overlaid.
with human prothrombin (Enzyme Research Laboratories). After incubation at 37°C, thrombin levels were quantified using Spectrozyme TH (American Diagnostics) and normalized to the area of the tissue section. Purified human α-thrombin (Enzyme Research Laboratories) was used as standard. PA assays were performed similarly to PT assays, with the following modifications: prothrombin was replaced by human Glu-plasminogen (Enzyme Research Laboratories), the standard curve was prepared using purified human plasmin (Enzyme Research Laboratories), and plasmin levels were measured using Spectrozyme PL (American Diagnostics). PT and PA activity are presented as fold-change relative to the levels measured in tissue samples collected from naïve mice.

**Measurements of immune parameters**

To assess responses in the peritoneal cavity, mice were euthanized and peritoneal exudate fluid was harvested by lavage using 5 ml saline. Neutrophil percentages were determined by inspection of Wright-Giemsa-stained cytospin smears. IL-6 and MCP-1 levels were determined by ELISA using OptEIA kits (BD Biosciences). Levels of hepatic mRNA encoding IL-1β, IL-6, IL-10, IFN-γ, and TNF-α were measured by real-time PCR; normalized to levels of GAPDH; and expressed as log10 fold-change relative to levels measured in uninfected control mice (3).

**Histology**

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E. The number of large pathological foci in each section was counted (i.e., areas with large numbers of infiltrating cells accompanied by evidence of anoxic or necrotic hepatocytes and/or bacterial colonization). Representative photomicrographs depict original magnification ×200.

**Statistics**

Statistical analyses were performed using the computer program Prism 4.0 (GraphPad Software). Survival data were analyzed by log-rank tests. Cytokine, cell numbers, and CFU data were analyzed by parametric tests. Student t test or one-way ANOVA, followed by Dunnett’s multiple comparison test, in which case mice were assigned a value of 100 CFU and data were analyzed by nonparametric tests. Box and whisker plots depict the minimum, maximum, median, and 25th and 75th percentile.

**Results**

**Fibrinogen impacts peritoneal inflammatory responses during yersiniosis**

Prior reports indicate fibrinogen can impact peritoneal inflammation after inoculation with Gram-positive bacteria (5). To assess roles for fibrinogen during Gram-negative infection, we infected fibrinogen-deficient and littermate control fibrinogen-heterozygous mice with *Y. enterocolitica* serotype O:8 strain WA (32). Specifically, we examined peritoneal exudate fluid collected 3 h after i.p. inoculation with 1 × 10^3 CFU *Y. enterocolitica*. The exudates obtained from fibrinogen-deficient mice, as compared with those collected from control fibrinogen-heterozygous mice, contained significantly reduced levels of the inflammatory cytokine IL-6 and chemokine MCP-1 (Fig. 1A, *p* = 0.006 and 0.0006, respectively). Fibrinogen-deficient mice also displayed significantly reduced numbers of neutrophils in exudate fluid (Fig. 1B, *p* = 0.0005).

At 3 h after inoculation with 1 × 10^3 CFU *Y. enterocolitica*, we could not detect bacteria in the peritoneal fluid. After increasing the challenge dose to 1 × 10^6 CFU, we observed significantly higher bacterial burden in fibrinogen-deficient mice as compared with littermate control fibrinogen-heterozygous mice (Fig. 1C, *p* = 0.0008). Fibrinogen deficiency also significantly increased the number of bacteria that reached the liver at 3 h after i.p. inoculation with 1 × 10^6 CFU *Y. enterocolitica* (Fig. 1C, *p* = 0.01). These data indicate that fibrin(ogen) impacts inflammation and host defense against bacteria at early times after i.p. inoculation with *Y. enterocolitica*.

**Fibrin protects against peritoneal yersiniosis**

*Y. enterocolitica* serotype O:8 is virulent in humans (34–38) and mice (32, 33). To investigate whether fibrinogen impacts susceptibility to *Y. enterocolitica* infection, we inoculated fibrinogen-deficient mice with a dose of *Y. enterocolitica* that is sublethal for wild-type mice. We observed that fibrinogen-deficient mice succumbed after inoculation with 0.05 LD50 *Y. enterocolitica* (2.5 × 10^3 CFU), whereas all fibrinogen-heterozygous littermate mice survived. The difference in survival was highly significant (*p* < 0.0001). Measurements of bacterial burden 5 d after *Y. enterocolitica* inoculation revealed significantly higher numbers of CFU in the livers and spleens of fibrinogen-deficient mice, as compared with control mice (Fig. 2B).

Because fibrinogen-deficient mice lack both fibrinogen and fibrin, next we treated wild-type mice with coumadin, a pharmaceutical used clinically for long-term anticoagulation. Coumadin-treated mice possess normal levels of circulating fibrinogen, but generate far less fibrin (4). Like fibrinogen-deficient mice, coumadin-
treated mice acutely succumbed to i.p. inoculation with \(2.5 \times 10^3\) CFU *Y. enterocolitica* (Fig. 2C). Coumadin treatment also significantly increased bacterial burden in livers and spleens collected 5 d after initiating the infection (Fig. 2D). The similar phenotypes observed in fibrinogen-deficient and coumadin-treated mice following i.p. inoculation with *Y. enterocolitica* strongly suggest that fibrin functions protectively during Gram-negative peritonitis.

**Fibrin functions protectively during systemic yersiniosis**

Prior reports suggest fibrin may physically trap bacteria, thereby limiting dissemination from the peritoneal cavity (6, 7) and liver vasculature (44). To investigate whether fibrin functions protectively beyond the peritoneal cavity during Gram-negative bacterial infection, we inoculated mice i.v. with *Y. enterocolitica*. Fibrinogen-heterozygous mice largely survived i.v. inoculation with \(1 \times 10^3\) CFU *Y. enterocolitica*, whereas fibrinogen-deficient mice displayed significantly reduced survival (Fig. 3A, \(p < 0.0001\)). Moreover, 5 d after i.v. inoculation with \(1 \times 10^3\) CFU *Y. enterocolitica*, fibrinogen-deficient mice displayed significantly increased bacterial burden in liver and spleen (Fig. 3B). As for i.p. inoculation, coumadin treatment also increased susceptibility to yersiniosis following i.v. inoculation with *Y. enterocolitica*: in comparison with untreated control mice, coumadin-treated mice exhibited significantly decreased survival (Fig. 3C, \(p < 0.0001\)) and increased bacterial burden (Fig. 3D). Coumadin treatment also led to significantly decreased survival when the initiation of treatment was delayed until 1 d after the initiation of infection (Fig. 3C, \(p = 0.002\)). Notably, coumadin treatment did not alter the levels of plasma fibrinogen measured at day 5 postinfection (data not shown). Together, these data suggest that fibrin not only activates peritoneal inflammation and antibacterial defense, but also contributes to antibacterial host defense mechanisms that operate beyond the peritoneal cavity. Interestingly, fibrinogen-deficient mice did not exhibit decreased survival when inoculated orally with *Y. enterocolitica* (\(1 \times 10^6\) CFU intragastrically; fibrinogen-heterozygous 25% survival, fibrinogen-deficient 29% survival, \(p > 0.05\)), suggesting that fibrin is critical for systemic, but not mucosal host defense against this Gram-negative bacterium.

**Kinetics of fibrin deposition during sublethal yersiniosis**

Given that fibrin-deficient mice displayed increased hepatic bacterial burden after inoculation with *Y. enterocolitica*, we investigated whether hepatic fibrin was detectable during the course of yersiniosis in wild-type mice. Specifically, we inoculated wild-type mice i.v. with \(1 \times 10^3\) CFU *Y. enterocolitica* and then analyzed bacterial burden and fibrin levels on days 1, 3, 5, 7, 10, and 14 postinfection. We detected hepatic bacteria in most mice on days 1, 3, 5, 7, and 10, with the greatest burden apparent on day 5 (Fig. 4A). Hepatic fibrin levels in mice infected with *Y. enterocolitica* were lower than those reported previously for mice infected with *Listeria* or *Toxoplasma* (3, 4). Whereas we could only detect fibrin in a subset of infected animals, nevertheless it was apparent that fibrin deposition peaked on day 5, thus coinciding with the time of maximal bacterial burden (Fig. 4B). Notably, yersiniosis also was characterized by significant thrombocytopenia (decreased platelet counts; Fig. 4C) and anemia (decreased hematocrits; Fig. 4D), suggesting systemic activation of coagulation and sublethal bleeding, respectively. Consistent with peak fibrin deposition on day 5, procoagulant activity also peaked at that time, as measured by in situ measurements of hepatic PT activity (Fig. 4E). Interestingly, hepatic PA activity peaked at day 3 and declined significantly by day 5, perhaps explaining the increased fibrin deposition on day 5 (Fig. 4F). Levels of D-dimer, a systemic measure of fibrinolysis, did not rise significantly above baseline at any time point (data not shown), but levels of hepatic mRNA encoding PAI-1 and TAFI, two inhibitors of fibrinolysis, increased over the course of yersiniosis (Fig. 4G, 4H), potentially explaining the decreased hepatic PA activity at the time of peak fibrin deposition. These data establish that hemostatic pathways become activated during sublethal yersiniosis in wild-type mice over the same period during which fibrinogen-deficient mice succumb to infection.

**Critical roles for TF, but not FXI, during yersiniosis**

Given evidence of coagulation during sublethal yersiniosis (i.e., increased hepatic PT activity and fibrin deposition), we investigated roles for key elements of the extrinsic and intrinsic coagulation pathways. TF is the primary initiator of the extrinsic
coagulation pathway. Although fully TF-deficient mice (i.e., mTF−/− mice) die in utero, TF function can be studied in vivo using mTF−/− mice expressing a human TF transgene (i.e., mTF−/−hTF+ mice) (42). Consistent with prior studies (42, 43), we observed that liver tissue from these low-TF mice contained significantly reduced levels of baseline hepatic PT activity (Fig. 5A, p < 0.0001). In contrast, baseline PT activity was similar in wild-type mice and mice deficient in FXI, a key component of the intrinsic coagulation pathway (Fig. 5B).

By day 5 after i.v. inoculation with 1 × 10^7 CFU Y. enterocolitica, levels of PT activity in low-TF mice had increased substantially, but still remained significantly below those measured in control mice (Fig. 5A, p < 0.0001). Like fibrinogen-deficient mice (Fig. 3), the infected low-TF mice also displayed significantly increased bacterial burden (Fig. 5C, p = 0.003) and reduced survival (Fig. 5D, p = 0.002) as compared with littermate control mTF−/− hTF+ mice. In contrast, the FXI-deficient mice did not exhibit increased bacterial burden or reduced survival (Fig. 5C, 5D). These data suggest that the TF-dependent extrinsic coagulation pathway plays a dominant role in the regulation of host-protective fibrin production during sublethal yersiniosis.

Synergistic protective roles for PAI-1 and TAFI during yersiniosis

Levels of hepatic mRNA encoding PAI-1 and TAFI increased over the course of yersiniosis, peaking on days 5 and 7, respectively (Fig. 4G, 4H). To investigate the importance of PAI-1 and TAFI expression, and to measure their capacity to regulate PA activity in this model, we infected gene-targeted mice lacking expression of PAI-1, TAFI, or both PAI-1 and TAFI. Basal levels of hepatic PA activity were similar in all these mice, and all the mice displayed similarly elevated PA activity at day 3 after i.v. inoculation with 1 × 10^7 CFU Y. enterocolitica (Fig. 5E). Consistent with our prior kinetic analysis (Fig. 4F), PA activity decreased in wild-type mice by day 5. In contrast, PA activity remained elevated on day 5 in mice deficient in PAI-1, TAFI, or both PAI-1 and TAFI, and these mice all exhibited significantly increased levels of hepatic PA activity when compared with wild-type mice (Fig. 5E, all p < 0.01). Levels of plasma D-dimer, a systemic marker of fibrinolysis, did not increase significantly in wild-type mice or TAFI-deficient mice infected with Y. enterocolitica. However, mice deficient in PAI-1 displayed significantly increased D-dimer levels on day 3 after inoculation with Y. enterocolitica, and PAI-1/TAFI-deficient mice displayed significantly increased D-dimer levels on both days 3 and 5 (Fig. 5F).

In comparison with wild-type mice, the bacterial burden in PAI-1–deficient mice increased significantly on both days 3 and 5 after inoculation with Y. enterocolitica, and the PAI-1/TAFI-deficient mice displayed even greater burden (Fig. 5G). TAFI-deficient mice exhibited bacterial burden similar to wild-type mice on days 3 or 5, but their splenic burden increased significantly on day 7 (Fig. 5G and data not shown). In comparison with wild-type mice, the TAFI–, PAI-1–, and PAI-1/TAFI-deficient mice all exhibited significantly reduced survival following inoculation with Y. enterocolitica; PAI-1/TAFI-deficient mice displayed the greatest susceptibility, followed sequentially by PAI-1–deficient mice and TAFI-deficient mice (Fig. 5H). Together, these findings suggest that PAI-1 and TAFI perform complementary functions that synergistically regulate fibrinolysis during yersiniosis.

Fibrinogen, TF, PAI-1, and TAFI impact inflammation and pathology during yersiniosis

After i.v. inoculation with Y. enterocolitica, fibrinogen-deficient mice, low-TF mice, and PAI-1/TAFI-deficient mice all displayed evidence of increased hepatic inflammation. Specifically, they displayed significantly increased levels of hepatic mRNA encoding...
IL-1β, IL-6, IL-10, IFN-γ, and TNF-α when compared with their respective control mice (Fig. 6 and data not shown). These observations must be interpreted with caution because the increased inflammation could be a consequence of the increased bacterial burden in these mice (Figs. 3, 5).

After infection with Y. enterocolitica, fibrinogen-deficient mice, PAI-1/TAFI-deficient mice, and low-TF mice all displayed significantly greater reductions in blood hematocrits and circulating platelet numbers when compared with control mice (Fig. 6). Whereas these hematological data were suggestive of bleeding, histological observations rarely revealed evidence of hemorrhage in fibrin-deficient mice and stool samples rarely tested positive for hemoglobin. This finding contrasts sharply with our prior studies of toxoplasmosis and listeriosis in fibrin(ogen)-deficient mice, in which the exacerbated anemia and thrombocytopenia were accompanied by frank hemorrhage (3, 4).

Although fibrin did not appear to protect from hepatic hemorrhage during yersiniosis, it did, nonetheless, dramatically impact liver histopathology (Fig. 7). Wild-type mice infected with Y. enterocolitica displayed very limited hepatic damage and small, sporadic sites of inflammation. Mice deficient in either PAI-1 or TAFI likewise displayed small foci of mixed leukocytes similar to those observed in wild-type mice. In striking contrast, fibrinogen-deficient mice, low-TF mice, and PAI-1/TAFI-deficient mice all displayed significantly exacerbated histopathology, evidenced by large bacterial colonies, and extensive areas of tissue damage characterized diffuse leukocyte infiltration and hepatocellular necrosis.

**Discussion**

This study demonstrates that fibrinogen-deficient mice and wild-type mice treated with coumadin succumb to doses of Y. enterocolitica that control mice survive. Moreover, fibrinogen-deficient mice, low-TF mice, and PAI-1/TAFI-deficient mice all exhibit similar decreases in survival, increases in bacterial burden, and exacerbations of anemia and hepatic histopathology after inoculation with Y. enterocolitica. These observations provide strong complementary evidence that fibrin performs critical protective functions in this model of sublethal Gram-negative infection. This study focused on fibrin, but we certainly acknowledge that other elements of hemostatic pathways also impact inflammation and infection (2, 12, 13, 45).

Our demonstration that fibrin can function protectively during Gram-negative infection, coupled with the wealth of evidence that excessive coagulation can function pathologically during sepsis (2), suggests that appropriate regulation of fibrin levels will be
critical for surviving certain septic infections. The upregulation of coagulation during infection is most likely initiated, at least in part, by host recognition of microbial components, leading to the activation of innate immunity, secretion of inflammatory cytokines, and consequent upregulation of TF expression, thus activating the extrinsic pathway of coagulation (2, 12, 14). Our data suggest that antagonistic targeting of the TF-dependent extrinsic pathway reduces fibrin levels to below those required for host defense against yersiniosis. Full antagonism of other critical components of the extrinsic coagulation pathway improves survival in mice and primates inoculated with another Gram-negative bacterium, *E. coli*, or its endotoxin (i.e., LPS) (2, 14, 21, 22). Thus, coagulation appears to be required for effective host defense against some, but not all, Gram-negative bacteria. This observation may help to explain why anticoagulant therapies that show great promise in preclinical models of *E. coli* sepsis often do not translate to human efficacy in clinical trials (1, 2, 29–31). One notable distinction between *E. coli* and *Y. pseudotuberculosis* is that the latter produces a relatively noninflammatory form of LPS when grown at 37°C (46). Additionally, host defense against *Y. pseudotuberculosis* is critically dependent upon T cells and IFN-γ (33), whereas T cells and IFN-γ actually contribute to pathology during *E. coli* infection (47, 48). A better understanding of the roles for coagulation during host defense and a full delineation of the pathogens that do and do not require protective coagulant responses may allow selective targeting of anticoagulant therapies to patients for whom the benefits of anticagulation outweigh the risks of suppressing fibrin-mediated host defense.
Another approach to preventing coagulopathy while maintaining fibrin-mediated host defense may be to target elements of DIC-related hemostatic pathways whose full antagonism does not deplete fibrin levels to below those required for protection. Our studies indicate that targeting TF overly compromises host defense against sublethal yersiniosis, whereas targeting FXI has no impact on survival, burden, or morbidity. FXI deficiency improves survival in the mouse model of lethal bacterial sepsis induced by cecal ligation and puncture (25), but does not improve survival during lethal Y. enterocolitica infection (data not shown), presumably because something other than FXI-mediated DIC is the primary cause of death during lethal yersiniosis. Together, these observations suggest that FXI does not contribute to antibacterial host defense, but sometimes contributes to septic coagulopathy. As
cannot exclude the possibility that fibrinolysis-independent impacts of TAFI play a role (20, 49), but our data decisively demonstrate increased fibrinolysis in TAFI-deficient mice infected with *Y. enterocolitica* (Fig. 5E, 5F). Renckens et al. (50) observed a transient increase in bacterial burden in TAFI-deficient mice infected with *E. coli*, but, in striking contrast to our results, this did not impair survival and, rather, appeared to protect against liver injury. Again, this apparent discrepancy may relate to intrinsic differences between the pathogenesis of *E. coli* and *Y. enterocolitica* infections. We also demonstrated that PAI-1 deficiency decreases survival and increases bacterial burden during yersiniosis. Renckens et al. (51) reported analogous observations for PAI-1–deficient mice infected with *Klebsiella pneumoniae*. As for TAFI, we cannot exclude the possibility that fibrinolysis-independent impacts of PAI-1 play a role (17), but our data decisively demonstrate increased fibrinolysis in PAI-1–deficient mice infected with *Y. enterocolitica* (Fig. 5E, 5F). Altogether, these studies indicate that full antagonism of either TAFI or PAI-1 can impair host defense against Gram-negative bacteria, but leave open the possibility that therapeutics targeting TAFI or PAI-1 may suppress coagulopathy without compromising host defense in certain settings (e.g., *E. coli* infection).

One of the most striking findings in this study was the synergy achieved by simultaneously antagonizing both TAFI and PAI-1. In comparison with mice deficient in either PAI-1 or TAFI, mice deficient in both PAI-1 and TAFI displayed significantly increased levels of plasma D-dimer and significantly exacerbated liver pathology. To our knowledge, this synergy has not been demonstrated previously in vivo, although it was postulated based upon in vitro models of thrombolysis (52). This synergy suggests that the consequences of targeting either PAI-1 or TAFI may be heavily influenced by the levels of the nontargeted factor. That is, the impact of PAI-1 antagonism may be far more pronounced in settings (e.g., certain tissues or disease states) in which TAFI levels are relatively low.

Multiple mechanisms appear to contribute to fibrin-mediated host defense against yersiniosis. The increased bacterial burden observed in fibrin-deficient mice infected with *Y. enterocolitica* resembles that previously reported by our laboratory (4), and others (5), after inoculating fibrin(ogen)-deficient mice and fibrinogen-mutant mice with Gram-positive bacteria. Thus, it seems that one fundamental function of fibrin is to limit bacterial growth. We are not aware of studies describing fibrin-binding proteins in *Y. enterocolitica*, but many other bacterial species possess diverse mechanisms for binding fibrin(ogen) and/or activating fibrinolysis (12, 13). It seems likely that bacteria have acquired the ability to interact with fibrin, at least in part, as a means to counter fibrin-mediated host defense.

Although previously it has been suggested that fibrin may physically prevent bacterial dissemination during peritoneal infections (6, 7), we observed that fibrin also impacts bacterial burden after i.v. delivery of *Y. enterocolitica*. One possible explanation is that fibrin may contribute to microvessel thrombosis within infected liver tissue, thereby physically preventing dissemination of bacteria from the bloodstream (44, 53). Alternatively, fibrin may restrain bacterial growth by facilitating the recruitment and activation of bactericidal phagocytes within infected tissues. Prior studies reported that fibrinogen activates peritoneal macrophages and neutrophils in mice inoculated with LPS (10) and Gram-positive bacteria (5). Consistent with those reports, this study demonstrates that fibrinogen facilitates the accumulation of peritoneal neutrophils after inoculation with *Y. enterocolitica* (Fig. 1). And consistent with prior studies of sterile peritonitis (10), this report demonstrates that fibrinogen stimulates production of fibrin.
MCP-1 and IL-6 at early time points after inoculation with Y. enterocolitica. These impacts may be mediated by CD11b/CD18 and/or TLR4, because these receptors reportedly activate cytokine and chemokine production via fibrinogen-mediated mechanisms (11, 54–56). MCP-1 recruits monocytes, and IL-6 is a pleiotropic cytokine known to promote fibrin deposition (57) and participate in host defense against Y. enterocolitica (58). Because monocytes express CD11b/CD18 and ligation of CD11b/CD18 by fibrinogen activates peritoneal phagocytes (5), it is easily conceivable that fibrin acts as an inducible matrix supporting feedback amplification of signals, leading to the activation and accumulation of antibacterial phagocytes at sites of infection.

Our findings suggest relatively minor roles for the hemostatic functions of fibrin during host defense against Gram-negative bacterial infection. Anemia, evidenced by reduced hematocrits, is evident in wild-type mice inoculated with sublethal doses of Y. enterocolitica (Fig. 4). Fibrin-deficient mice show exacerbated anemia (Fig. 6) and begin to succumb to yersiniosis at day 5 of the infection, which coincides with the time of maximal anemia in wild-type mice (Fig. 3). We likewise observed exacerbated anemia in our prior studies of fibrin-deficient mice inoculated with protozoa and Gram-positive bacteria (3, 4). In those studies, we observed unmistakable evidence of frank hemorrhage in the livers of fibrinogen-deficient mice, leading us to conclude that the anemia reflected bleeding. In contrast, we observed relatively little evidence of hepatic hemorrhage in fibrin-deficient mice during yersiniosis, despite a high bacterial burden (Fig. 7). Thus, the extent to which fibrin provides critical hemostatic protection during infection appears to vary, presumably reflecting the extent to which different pathogens predispose to hemorrhagic pathology.

Although hemorrhage was uncommon in the hepatic lesions of Y. enterocolitica-infected fibrin-deficient mice, we nevertheless observed significant differences in the overall appearance of these lesions in comparison with those observed in fibrin-sufficient mice. Specifically, fibrin-deficient mice exhibited severe hepato-cellular necrosis. We also observed increased levels of inflammatory cytokines in the liver at later stages of septic yersiniosis in fibrin-deficient mice. Increased cytokine production also is evident in fibrinogen-deficient mice during septic listeriosis (4). Many mechanisms could potentially account for the exacerbation of hepatic pathology and inflammation in the absence of fibrin; however, we believe it most likely to be a consequence of the increased bacterial burden in fibrin-deficient mice.

Septic coagulopathy can be caused by many different types of microorganisms (28, 59). Ideally, identical therapeutics and dosing regimens would be appropriate for all patients presenting with septic coagulopathy, regardless of the causative microbe. The development of universal therapies may be confounded by the fact that microorganisms interact with components of the coagulation and fibrinolytic pathways in diverse and unique ways (12, 13). Moreover, the antibacterial functions of fibrin-mediated host defense may be dispensable in septic patients when suitable antibiotics are prescribed, but may be critical in cases in which the causative pathogens are insensitive to antibiotics. Likewise, fibrin-mediated hemostatic defense may only be critical for some pathogens. The rational design of universally effective sepsis therapeutics, if feasible, should be aided by a precise understanding of the regulation of host-protective fibrin in diverse models of infection.

In summary, this report demonstrates that fibrin performs multiple host defense functions during yersiniosis. In this model of Gram-negative infection, fibrin suppresses bacterial burden, influences inflammation and pathology, and can be critical for survival. The balance of coagulation and fibrinolysis dictates levels of protective fibrin during Y. enterocolitica infection. Thus, the mouse model of yersiniosis provides a means to investigate the relative importance of specific hemostatic pathway elements during fibrin-mediated host defense.

Acknowledgments
We thank Lawrence Johnson for critical reading of this manuscript, Deborah Duso for technical assistance, and the employees of Trudeau Institute’s Animal Facilities for dedicated breeding and care of the mice used in these studies.

Disclosures
The authors have no financial conflicts of interest.

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