THROMBOSIS AND HEMOSTASIS

Mice expressing a mutant form of fibrinogen that cannot support fibrin formation exhibit compromised antimicrobial host defense

Joni M. Prasad,[1] Oleg V. Gorkun,[2] Harini Raghu,[1] Sherry Thornton,[3] Eric S. Mullins,[4] Joseph S. Palumbo,[4] Ya-Ping Ko,[5] Magnus Höök,[5] Tovo David,[6] Shaun R. Coughlin,[6] Jay L. Degen,[1] and Matthew J. Flick[1]

[1]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; [2]Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC; [3]Division of Rheumatology, [4]Division of Hematology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; [5]Center for Infectious and Inflammatory Diseases, Institute for Biosciences and Technology, Texas A&M Health Science Center, Houston, TX; and [6]Cardiovascular Research Institute, University of California at San Francisco, San Francisco, CA

Key Points

- Mutation of the fibrinogen Aα chain in mice to selectively eliminate thrombin cleavage prevents fibrin polymer formation in vivo.
- Fibrin polymer formation drives antimicrobial function and supports host survival following *S aureus* peritoneal infection.

Fibrin(ogen) is central to hemostasis and thrombosis and also contributes to multiple physiologic and pathologic processes beyond coagulation. However, the precise contribution of soluble fibrinogen vs insoluble fibrin matrices to vascular integrity, tissue repair, inflammation, and disease has been undefined and unapproachable. To establish the means to distinguish fibrinogen- and fibrin-dependent processes in vivo, Fib^{AEK} mice were generated that carry normal levels of circulating fibrinogen but lack the capacity for fibrin polymer formation due to a germ-line mutation in the Aα chain thrombin cleavage site. Homozygous Fib^{AEK} mice developed to term and exhibited postnatal survival superior to that of fibrinogen-deficient mice. Unlike fibrinogen-deficient mice, platelet-rich plasma from Fib^{AEK} mice supported normal platelet aggregation in vitro, highlighting that fibrinogen^{AEK} retains the functional capacity to support interactions with platelets. Thrombin failed to release fibrinopeptide-A from fibrinogen^{AEK} and failed to induce polymer formation with Fib^{AEK} plasma or purified fibrinogen^{AEK} in 37°C mixtures regardless of incubation time. Fib^{AEK} mice displayed both an absence of fibrin polymer formation following liver injury, as assessed by electron microscopy, and a failure to generate stable occlusive thrombi following $FeCl_3$ injury of carotid arteries. Fib^{AEK} mice exhibited a profound impediment in *Staphylococcus aureus* clearance following intraperitoneal infection similar to fibrinogen-deficient mice, yet Fib^{AEK} mice displayed a significant infection dose-dependent survival advantage over fibrinogen-deficient mice following peritonitis challenge. Collectively, these findings establish for the first time that fibrin polymer is the molecular form critical for antimicrobial mechanisms while simultaneously highlighting biologically meaningful contributions and functions of the soluble molecule. (*Blood.* 2015;126(17):2047-2058)

Introduction

Fibrin(ogen) is a key factor in the control of blood loss and the development of potentially fatal venous or arterial thrombotic events (eg, deep vein thrombosis, pulmonary embolism, myocardial infarction, and stroke). Fibrin(ogen) is also instrumental in reparative and protective inflammatory processes, but exuberant or persistent fibrin(ogen) is associated with many diseases, including cancer, vessel wall disease, and inflammatory diseases.[1-4] Polymer is often presumed to be the key structural form of the molecule coupled to fibrinogen-dependent physiologic and pathologic processes in vivo, but resolving the precise contributions of soluble fibrinogen and fibrin in vivo has been formally problematic. The uncertainty is underscored by the known potential for soluble fibrinogen to support important functions, including the capacity of the soluble, circulating molecule to support integrin $\alpha_{IIb}\beta_3$-mediated platelet aggregation/thrombus formation. Similarly, leukocyte engagement of immobilized fibrinogen in vitro through integrin[5-7] and nonintegrin[8,9] receptors is thought to support cell adhesion, migration, phagocytosis, nuclear factor-κB–mediated transcription, chemokine and cytokine elaboration, degranulation, and other processes.[9-13] Both fibrinogen and fibrin may have distinct and specialized properties that direct thrombotic and/or inflammatory events in vivo, but the precise form of the molecule driving fibrin(ogen)-associated events has not been established.

Host fibrin(ogen) is a known determinant of infection outcome for many bacterial pathogens (eg, *Staphylococcus aureus*, *Yersinia pestis,* and *Streptococcus pyogenes*). Depending on the context, fibrin(ogen) appears to support either microbial virulence or host antimicrobial defense and potentially both via different mechanisms. For example, the elimination of host fibrin(ogen) significantly reduced the virulence of *S aureus* in the context of an intravenous infection challenge.[14] In contrast, in studies of *S aureus* peritonitis, fibrin(ogen) deficiency favored the virulence of the pathogen by impeding the rapid clearance of bacteria in the peritoneal cavity.[15,16] Similar studies using mice with a genetically imposed reduction in circulating prothrombin or pharmacologic inhibition of thrombin activity also resulted in

Submitted April 9, 2015; accepted July 26, 2015. Prepublished online as *Blood* First Edition paper, July 30, 2015; DOI 10.1182/blood-2015-04-639849.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

significantly compromised *S aureus* clearance from the peritoneal cavity.[17,18] Such findings are consistent with, but do not prove, fibrin polymer as a critical molecular feature of the host antimicrobial response following *S aureus* peritoneal infection. The benefits and/or liabilities to the host and pathogen of the 2 molecular forms of host fibrin(ogen) remain an open question.

To establish an experimental system that provides the means to formally resolve the biologic contributions of fibrin and fibrinogen in any physiologic and pathologic process in vivo, we generated knock-in mice (termed Fib^{AEK} mice) in which the Aα chain of fibrinogen was selectively mutated to eliminate thrombin-mediated removal of fibrinopeptide A (FpA). Here, we report the phenotypic consequences for mice carrying normal levels of fibrinogen that is "locked" in the soluble, monomeric form with respect to development, reproductive success, hemostatic capacity, and clotting function both in vitro and in vivo. Furthermore, the power of Fib^{AEK} mice to resolve whether fibrinogen or fibrin is the molecular form of host fibrin(ogen) central to biologic outcome is exemplified through studies of fibrin(ogen)-mediated host defense in *S aureus* peritonitis.

Materials and methods

Generation of Fib^{AEK} gene-targeted mice

Details of gene targeting in mouse embryonic stem cells and of the generation of Fib^{AEK} mice can be found in the supplemental Materials and Methods available on the *Blood* Web site.

Analysis of Fib^{AEK} expression and hematologic profiles

Analyses of gene expression and hematological profile were performed as described in the supplemental Materials and Methods.

Purification and analysis of fibrinogen protein

Fibrinogen was purified from citrate-plasma and analyzed as described in the supplemental Materials and Methods.

Fibrin polymerization analysis

Fibrin polymerization analyses were performed as described in the supplemental Materials and Methods.

Comparative real-time analyses of thrombus formation by intravital microscopy

$FeCl_3$ injury and analysis of the left carotid artery of mice were performed as described in the supplemental Materials and Methods.

Liver puncture injury and scanning electron microscopy

Needle puncture injury to the right medial lobe of the liver was performed and analyzed as described in the supplemental Materials and Methods.

Flow cytometry analysis of resident peritoneal cells

Flow cytometry-based phenotyping analyses of cells isolated by peritoneal lavage were performed as described in the supplemental Materials and Methods.

ClfA-dependent S aureus aggregation and coagulase-induced plasma clots

The capacity of purified $fibrinogen^{AEK}$ to support bacterial aggregation in vitro with wild-type $ClfA^+$ *S aureus* and participate in coagulase-induced polymerization was established as described in the supplemental Materials and Methods.

S aureus-induced peritonitis

Staphylococcus aureus strain Newman (kindly provided by T. J. Foster from Trinity College) and strain USA300 (obtained from ATCC) were used in infection studies as described in the supplemental Materials and Methods.

Results

Site-directed mutagenesis of the endogenous fibrinogen Aα chain gene

To generate mice carrying a mutant form of fibrinogen with no capacity for thrombin-mediated release of FpA, the Aα chain thrombin cleavage site residues $E^{P6}GGGVR^{P1}$ were converted to $A^{P6}DDDDK^{P1}$ (Figure 1A-B). The objective of this sequence change was to render the Aα chain completely insensitive to thrombin-mediated proteolysis, but allow for in vitro biochemical evaluation of enterokinase (EK)-dependent release of FpA.[19] The choice of generating an EK cleavage site was based on the unusual sequence specificity of EK (P4-P1 residues DDDDK) with the distinct importance of the substrate residues upstream of the cleavage site but not the downstream residues (P1′-P3′; which must remain Gly-Pro-Arg to form a polymer in fibrin monomer), the complete lack of any DDDDK sequences in the 3 fibrinogen chains, and the fact that endogenous EK is a membrane-associated serine protease expressed only in the duodenum[20]; thus, barring traumatic injury to the duodenum, the enzyme would not be expected to encounter fibrinogen in vivo. Identification of homologous recombination for the Aα chain targeting vector, the AEK mutation, and *Cre* recombinase-mediated deletion of the HPRT selectable marker was confirmed by polymerase chain reaction (PCR) of genomic DNA (representative data in Figure 1C). Expression of the mutant allele was readily detected by reverse transcriptase (RT)-PCR analysis of hepatic mRNA isolated from adult mice (Figure 1D). Steady-state plasma fibrinogen levels (Figure 1E), as well as the size and integrity of individual chains of purified fibrinogen (Figure 1F), were similar in adult homozygous Fib^{AEK} and wild-type (WT) mice.

Fib^{AEK} blood fails to support thrombin-induced clot formation but can support platelet aggregation in vitro

The capacity of thrombin to induce polymer formation for $fibrinogen^{AEK}$ was first evaluated in whole blood clotting assays. Coagulation of whole blood from WT mice was rapid in reactions initiated by the addition of either thrombin or thromboplastin (mean values of 17.4 and 19.3 seconds, respectively; Figures 2A-B). However, clot formation in whole blood from homozygous Fib^{AEK} mice was not detected following addition of either thrombin (Figure 2A) or thromboplastin (Figure 2B). Consistent with these findings, in all standard plasma coagulation tests where fibrin clot formation is the endpoint [i.e., prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT)], samples from Fib^{AEK} mice failed to clot regardless of observation period at 37°C, whereas plasma from WT mice had average clotting times of 12.5, 29.3, and 14.9 seconds for the PT, aPTT, and TT (Table 1). Complementary whole blood studies were done over an 18-hour period at 37°C focusing on both clotting and platelet-mediated clot retraction. Under conditions where WT whole blood clots formed in just seconds and clot retraction became overtly evident within minutes, clot formation or retraction was not observed in any of the samples from Fib^{AEK} mice (Figure 2C). Even 18 hours after thromboplastin addition, neither clot formation nor retraction could be appreciated in Fib^{AEK} whole blood samples; the only observable event was red cell sedimentation. Nevertheless, the overall structural integrity of circulating $fibrinogen^{AEK}$ was strongly inferred by the capacity of Fib^{AEK} platelet-rich plasma, but not

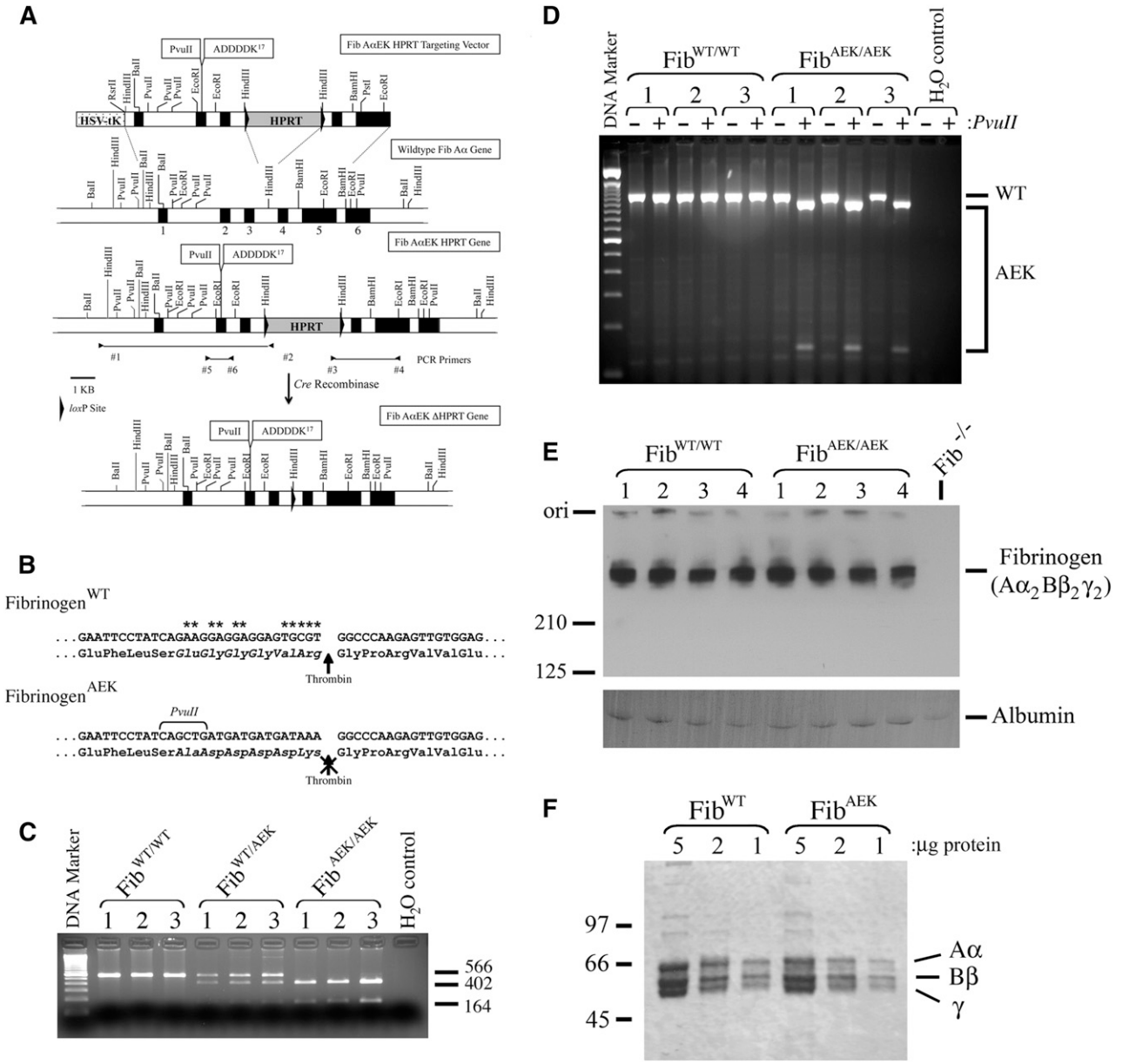

Figure 1. Generation of Fib^{AEK} mice that express normal levels of a mutant form of fibrinogen with a mutation in the fibrinopeptide sequence of the Aα chain immediately upstream of the thrombin cleavage site. (A) Schematic diagram of the gene targeting strategy for inserting 11 nucleic acid substitutions into the endogenous fibrinogen Aα-chain gene of mouse embryonic stem (ES) cells. Black boxes symbolize gene exons. Arrowheads indicate relative positions of nucleotide primers used for PCR-based genotyping. Note that ES cell clones were screened for incorporation of the Fib AαEK HPRT targeting vector by homologous recombination, and deletion of the HPRT minigene was accomplished by crossing mice carrying the targeted allele to transgenic mice expressing *Cre* recombinase under the control of the cytomegalovirus (CMV) promoter. (B) Summary of the nucleic acid substitutions, and resulting amino acid changes, for the mutated fibrinogen Aα-chain gene of Fib^{AEK} mice. Asterisks highlight positions of the nucleotide substitutions. (C) Representative PCR analyses to establish animal genotypes using DNA template from ear biopsies of WT, heterozygous, and homozygous mutant Fib^{AEK} mice. Primers 5 and 6 were used to amplify a 566-bp fragment, which was subsequently digested with *Pvu*II to yield the diagnostic fragments of 402 and 164 bp. (D) RT-PCR analysis of total hepatic RNA isolated from each of 3 individual WT and Fib^{AEK} homozygous mice. Primers specific to sequences within exons 1 and 5 were used to generate an 1185-bp PCR product. The product generated from a WT transcript was insensitive to cleavage by *Pvu*II, whereas the product generated from the mutant Fib^{AEK} transcript was cleaved into expected 1047- and 138-bp fragments. (E) Western blot analysis of plasma (nonreducing conditions) harvested from 4 WT mice, 4 Fib^{AEK} mice, and 1 Fib$^{-/-}$ mouse using fibrinogen-directed polyclonal antisera. (F) Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gel (reducing conditions) showing affinity-purified fibrinogen preparations from WT and Fib^{AEK} mice. The positions of the Aα, Bβ, and γ chains are indicated.

Fib$^{-/-}$ platelet-rich plasma, to support platelet aggregation following ADP stimulation (Figure 2D).

Fib^{AEK} mice exhibit a survival advantage over Fib$^{-/-}$ mice but cannot tolerate the challenge of pregnancy

Crosses between heterozygous Fib^{AEK} mice revealed that a significant fraction, but not all, of expected homozygous Fib^{AEK} mice survived to weaning (~3 weeks of age). Of the first 193 pups generated from heterozygous breeding pairs, 51 (26%) were WT (WT/WT), 113 (58.6%) were heterozygous (WT/AEK), and only 29 (15%) were homozygous mutant (AEK/AEK). The partial loss of homozygous Fib^{AEK} offspring was typically associated with spontaneous perinatal abdominal hemorrhagic events and soft tissue bleeds similar to those previously reported in fibrinogen- and prothrombin-deficient neonates.[21-23] However, homozygous Fib^{AEK} offspring surviving the perinatal

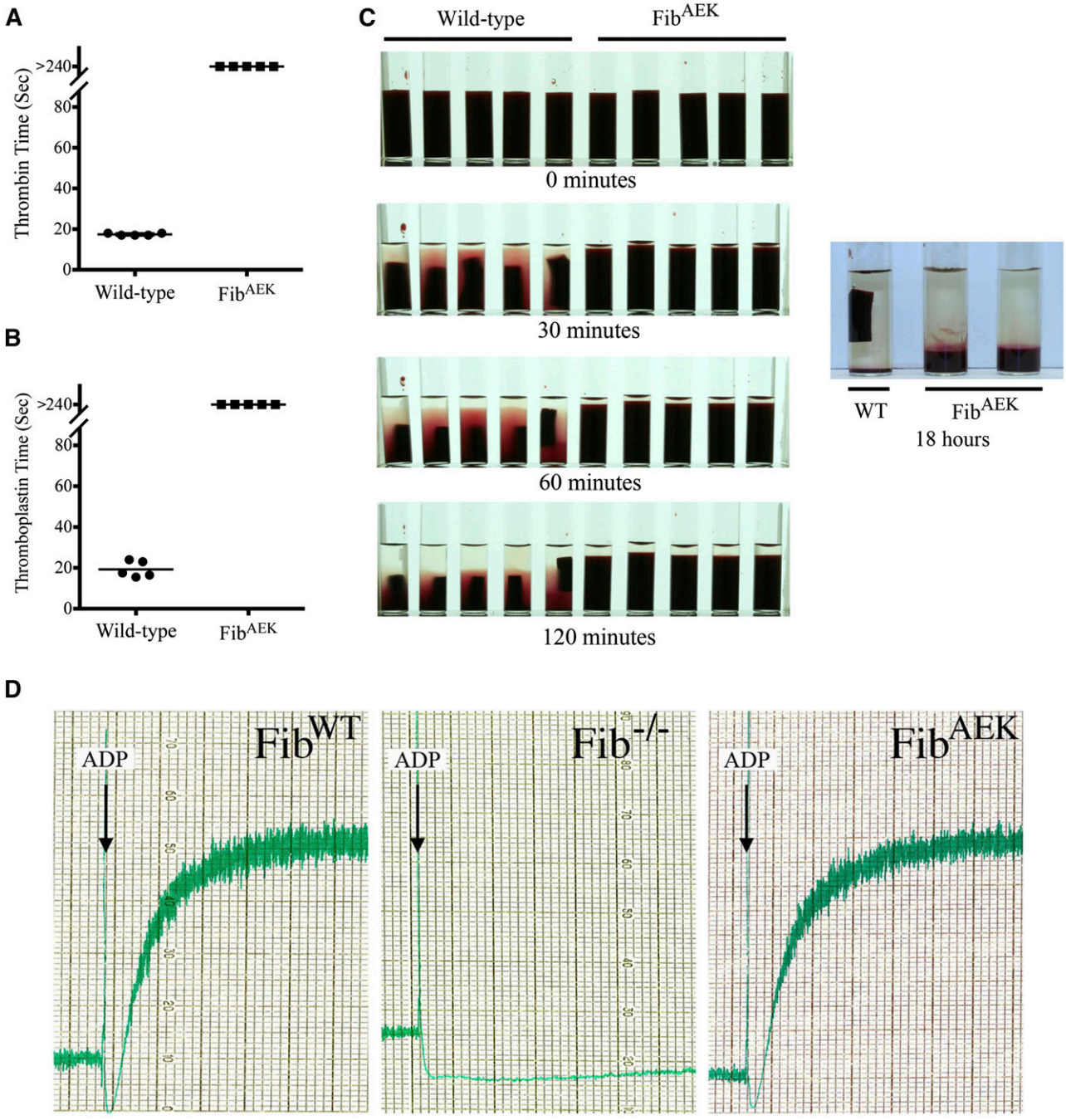

Figure 2. Whole blood isolated from Fib^{AEK} mice does not clot following the addition of thrombin or thromboplastin but supports platelet aggregation similar to WT fibrinogen. Clotting times for whole blood from each of 5 WT and Fib^{AEK} mice were determined following the addition of 2 U/mL (A) thrombin or (B) thromboplastin. The horizontal bars indicate average clotting times (seconds) for the groups. Note that clots were never detected for whole blood isolated from Fib^{AEK} mice following the addition of thrombin or thromboplastin. $P < .001$ by Fisher's exact test for each analysis. (C) Qualitative analysis of whole blood clots and clot retraction following the addition of thromboplastin. Retracted clots were readily observed within 30 minutes for samples of whole blood isolated from WT mice; whereas even up to 18 hours following addition of thromboplastin, no clot of any form could be appreciated in whole blood samples isolated from Fib^{AEK} mice. (D) Platelet aggregation analysis using platelet-rich plasma isolated from WT, Fib$^{-/-}$, and Fib^{AEK} mice. For each analysis, aggregation was initiated using the platelet agonist ADP.

period generally survived well into adulthood in the absence of other challenges. To formally compare the perinatal survival profile of homozygous Fib^{AEK} mice and homozygous fibrinogen-deficient (Fib$^{-/-}$) mice within precisely the same genetic background and microenvironment, an analysis was performed using C57Bl/6-inbred breeding pairs made up of homozygous males and heterozygous females. Here, a significantly higher fraction of homozygous Fib^{AEK} offspring survived to weaning relative to Fib$^{-/-}$ offspring (Table 2). Heterozygous Fib^{AEK} female mice consistently carried litters to term without hemorrhagic consequences, but homozygous Fib^{AEK} female mice were unable to sustain pregnancies. Ten of 10 homozygous Fib^{AEK} mice impregnated by Fib^{AEK} homozygous males died or became moribund in midgestation (E9.5-E10.5), often with evidence of overt bleeding. Histologic analysis of uterine tissue from pregnant homozygous Fib^{AEK} mice revealed massive intrauterine hemorrhage characterized by free maternal blood cells (evidenced by enucleated red

Table 1. Hematologic profile of Fib^{AEK} mice

	Fib^{WT} (N = 6)	Fib^{AEK} (N = 6)
WBC ($\times 10^9$/L)	4.95 ± 1.7	4.79 ± 1.8
RBC ($\times 10^{12}$/L)	8.92 ± 0.5	9.02 ± 0.5
Hemoglobin (g/dL)	12.15 ± 0.7	12.23 ± 0.5
Hematocrit (%)	51.52 ± 3.7	50.80 ± 2.9
Platelets ($\times 10^9$/L)	1005 ± 157	892 ± 145
PT (seconds)	12.5 ± 0.2	>180
aPTT (seconds)	29.3 ± 2.7	>300
Thrombin time (seconds)	14.9 ± 1.1	>90

blood cells) within the uterus and placental tissue (supplemental Figure 1). Notably, the developing homozygous Fib^{AEK} embryos appeared developmentally sound, with no evidence of free embryonic (nucleated) red blood cells (supplemental Figure 1).

Fibrinogen^{AEK} fails to polymerize following incubation with thrombin but polymerization can be induced by enterokinase in vitro

Using standard turbidity assays, plasma prepared from WT mice displayed a typical thrombin-induced fibrin assembly profile (Figure 3A). In contrast, no change in turbidity was detected following the addition of thrombin to plasma prepared from Fib^{AEK} mice, even at 40 times higher thrombin concentrations (Figure 3A). Consistent with the genetically imposed substitution of an enterokinase cleavage site in place of the thrombin cleavage site, plasma prepared from Fib^{AEK}, but not plasma from Fib^{WT} mice, exhibited a change in turbidity profile consistent with polymer formation following addition of enterokinase (Figure 3B). This turbidity change followed a typical lag phase but occurred over many hours (reflecting the relatively low enterokinase levels used). Reaction mixtures of purified fibrinogen preparations produced a similar pattern to that observed with plasma following the addition of thrombin or enterokinase (Figure 3C-D, respectively). Scanning electron microscopic analyses directly established that overall morphology of fibrin polymers generated with enterokinase-cleaved fibrinogen^{AEK} was similar to those generated with thrombin-cleaved WT fibrinogen, with the exception that the fibers in the former seemed generally thicker (Figure 3E, far right and far left). Diffuse and thin macromolecular structures were observed in reactions of fibrinogen^{AEK} and thrombin, perhaps representing thrombin-mediated fXIII (which copurifies with fibrinogen) activation and subsequent fibrinogen cross-linking (Figure 3E). Only comparatively small aggregates, potentially an artifact of processing for scanning electron microscopy, were observed in reactions of WT fibrinogen with EK enzyme.

Fibrinopeptide A of fibrinogen^{AEK} cannot be released by thrombin

Thrombin-mediated fibrinopeptide release was compared using purified mutant and WT fibrinogen. A time-dependent molecular weight shift in the fibrinogen Aα chain (corresponding to the α chain without FpA) was observed with WT fibrinogen following thrombin addition, whereas only the intact Aα chain was observed for fibrinogen^{AEK} reactions regardless of incubation time with thrombin (Figure 4A). Even fibrinogen^{AEK} reactions with exceptionally high concentrations of thrombin (eg, 200 nM) over extensive incubation times (>30 minutes) revealed no proteolytic conversion of the Aα chain to the α chain (data not shown). The absence of FpA release was confirmed by high-performance liquid chromatography (HPLC) analysis of peptides generated in fibrinogen/thrombin incubation mixtures. Thrombin-released FpA from WT fibrinogen was detected on chromatograms within 1 minute (Figure 4B). However, thrombin-mediated FpA release was never detected with fibrinogen^{AEK} (ie, FpA-AEK) regardless of the incubation time (Figure 4B).

A time-dependent release of FpB from the Bβ chain was maintained following thrombin addition with both fibrinogen^{WT} and fibrinogen^{AEK} (Figure 4A-B). However, consistent with findings with other fibrinogen variants,[24-28] HPLC analyses suggested that FpB release from fibrinogen^{AEK} was delayed relative to thrombin-mediated release of FpB from WT fibrinogen (Figure 4B-C). The calculated specificity constants (k_{cat}/K_M) confirmed a significant diminution in the efficiency of FpB release from fibrinogen^{AEK} relative to WT fibrinogen (Figure 4D).

Fib^{AEK} mice exhibit compromised hemostasis and protection from occlusive thrombus formation following acute challenge

To evaluate the ability of Fib^{AEK} mice to control blood loss following acute vessel injury, we compared tail-bleeding times in cohorts of WT, Fib^{AEK}, Fib$^{-/-}$, and Fib$^{+/-}$ mice. As shown in Figure 5A, WT and Fib$^{+/-}$ mice rapidly and uniformly stopped bleeding following tail tip amputation, whereas Fib^{AEK} and Fib$^{-/-}$ mice exhibited a major, albeit not identical, impediment in the control of blood loss. Unlike Fib$^{-/-}$ mice, which uniformly failed to stop blood loss over the entire observation period (>6 minutes), half of Fib^{AEK} mice analyzed ultimately stopped blood loss within this timeframe (Figure 5A). Thus, although hemostasis is compromised in Fib^{AEK} mice, these animals retain an advantage over mice with no fibrinogen in their capacity to control blood loss.

In complementary analyses, WT and Fib^{AEK} mice were challenged with $FeCl_3$-induced carotid artery injury and time to occlusion was tracked using a Doppler flow probe (Figure 5B). WT mice developed a complete and sustained carotid vessel occlusion in an average time of 16 minutes. In contrast, the majority (9 of 11) of the Fib^{AEK} mice challenged never displayed complete vessel occlusion during the observation period (>35 minutes). The Doppler flow tracings for Fib^{AEK} mice suggested embolization events in all but 1 Fib^{AEK} animal (data not shown). Stable occlusion occurred in 2 Fib^{AEK} mice, but over a significantly longer timeframe than for any of the WT mice (ie, 28 and 29 minutes). Thus, the failure of thrombin-mediated fibrin polymer formation documented with fibrinogen^{AEK} in vitro results in compromised hemostasis and resistance to occlusive thrombus formation in vivo.

Fib^{AEK} mice display a lack of extravascular fibrin deposition following liver injury

To directly interrogate the capacity of Fib^{AEK} mice to generate extravascular fibrin polymer in an in vivo setting, we challenged WT, Fib$^{-/-}$, and Fib^{AEK} mice with a liver needle puncture injury and evaluated clot formation in the damaged zone using scanning electron microscopy. Fibrin polymer was readily visualized in the damaged zone of every WT mouse, with some fields having substantial numbers

Table 2. Analysis of postnatal survival comparing offspring with complete fibrinogen deficiency (Fib$^{-/-}$) and mice homozygous for Fib^{AEK}

	♀Fib$^{WT/Null}$ × ♂Fib$^{Null/Null}$		♀Fib$^{WT/AEK}$ × ♂Fib$^{AEK/AEK}$	
Offspring genotype	Fib$^{WT/-}$	Fib$^{-/-}$	Fib$^{WT/AEK}$	Fib$^{AEK/AEK}$
Mendelian ratio	1	1	1	1
No. observed (at 3 weeks)	170	85	130	93
% of expected*	100%	50%	100%	72%

*$P < .05$, χ^2 analysis.

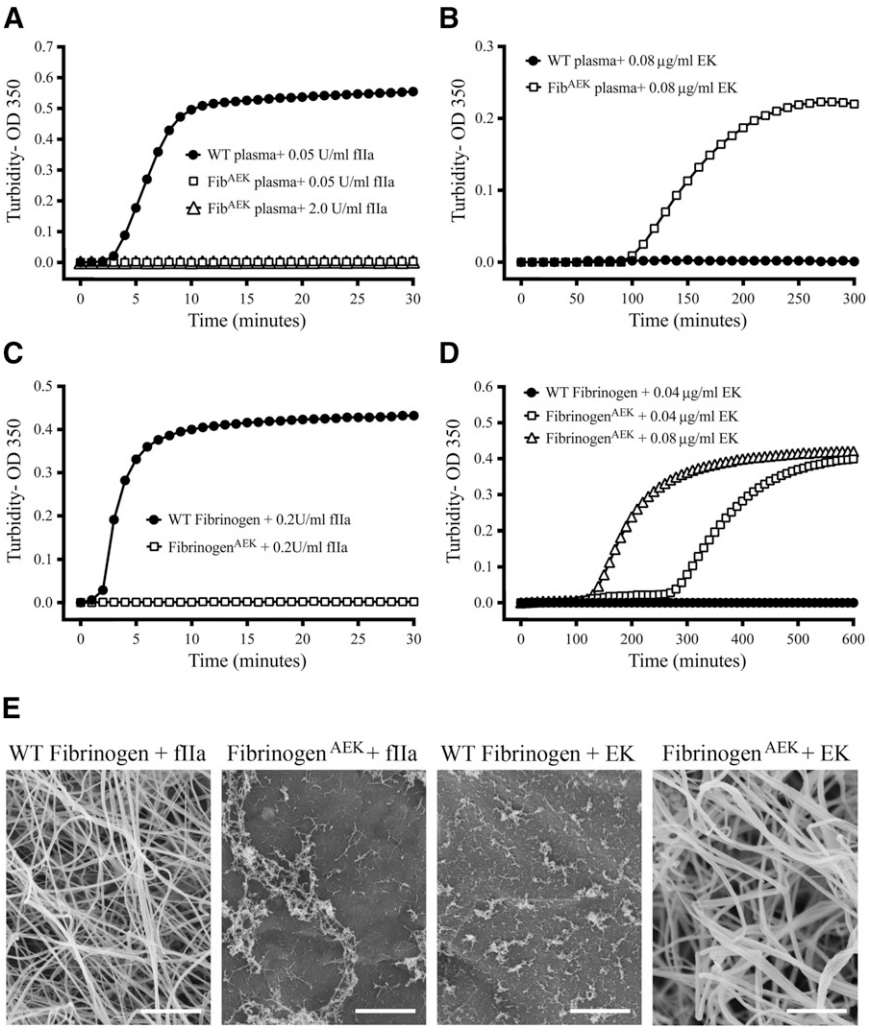

Figure 3. Fibrinogen^{AEK} forms a polymer following incubation with enterokinase enzyme but not thrombin. Representative turbidity analyses using plasma isolated from WT (closed circles) and Fib^{AEK} (open symbols) mice following incubation with (A) thrombin or (B) enterokinase enzyme. Similar representative turbidity analyses using purified WT fibrinogen (closed circles) and fibrinogen^{AEK} (open symbols) following incubation with (C) thrombin or (D) enterokinase enzyme. The purified fibrinogen concentration in each analysis was 0.04 mg/mL. To prevent spurious thrombin activity, reactions with enterokinase enzyme included the addition of the specific thrombin inhibitor lepirudin at 0.025 mg/mL. (E) Scanning electron micrographs of products formed from reaction mixtures of purified WT fibrinogen or fibrinogen^{AEK} with thrombin or enterokinase. Scale bar, 2.5 μm.

of platelets associated with the polymer and other areas revealing primarily polymers rich in red blood cells (representative views in Figure 6, left). Fib$^{-/-}$ mice challenged in parallel displayed a total absence of polymer within the injured zones. Here, fields within the injured zone of Fib$^{-/-}$ livers were rich in platelet clusters or free red blood cells (representative views in Figure 6, center). Importantly, studies of Fib^{AEK} mice established a distinct absence of any extravascular fibrin polymer following injury (representative views in Figure 6, right). Indeed, the injured hepatic zones in Fib^{AEK} mice were essentially indistinguishable from those observed in Fib$^{-/-}$ mice (Figure 6), despite the fact that Fib^{AEK} animals retain normal circulating levels of fibrinogen.

Fib^{AEK} mice fail to efficiently clear *S aureus* introduced into the peritoneal cavity, but retaining soluble fibrinogen offers a context-dependent host survival advantage in acute peritonitis

As a first illustration of the uniquely instructive nature of Fib^{AEK} mice, we sought to directly test the hypothesis that polymer formation is key to the implementation of the fibrinogen-dependent antimicrobial response in *S aureus* peritonitis challenge. Multiple prior reports have indicated that fibrin(ogen) is critical for a rapid and robust clearance of *S aureus* from the peritoneal cavity following an intraperitoneal infection.[15,16] However, the precise mechanism, and whether this host antimicrobial response was fibrinogen- or fibrin-dependent, has remained unknown. Consistent with previous reports,[16,18] WT mice challenged with an intraperitoneal injection of ~10^9 colony-forming units (CFUs) of either the clinical isolate methicillin-resistant *S aureus* (MRSA) strain USA300 (Figure 7A) or strain Newman WT *S aureus* (Figure 7B) eliminated ~99% of the bacteria within 1 hour based on analyses of peritoneal lavage fluid. In contrast, ~15-fold higher USA300 CFUs were retrieved by peritoneal lavage of Fib^{AEK} mice and virtually the entire initial CFU inoculum of USA300 was retrieved from Fib$^{-/-}$ mice (Figure 7A). Similarly, the same numbers of strain Newman CFUs were retrieved by peritoneal lavage of Fib^{AEK} and Fib$^{-/-}$ mice as were present in the initial infection volume (Figure 7B). Collectively, the results indicate that the ability to form fibrin in WT and Fib$^{+/-}$ mice significantly contributes to rapid bacteria clearance in the peritoneal cavity.

To begin to determine the molecular or cellular basis for the impressive difference in peritoneal bacteria clearance between WT and Fib^{AEK} animals, analyses of resident peritoneal cells were performed. Both the number and distribution of myeloid (Figure 7C) and lymphoid (supplemental Figure 2) resident peritoneal cells found in unchallenged WT and Fib^{AEK} mice were indistinguishable. Cytospin preparations of lavage fluid from challenged Fib^{AEK} mice and Fib$^{-/-}$ mice confirmed a persistence of large numbers of free bacteria in the peritoneal cavity of these animals, whereas preparations from WT or Fib$^{+/-}$ mice were largely devoid of bacteria (Figure 7D). In vitro analyses indicated that both WT fibrinogen and fibrinogen^{AEK} were

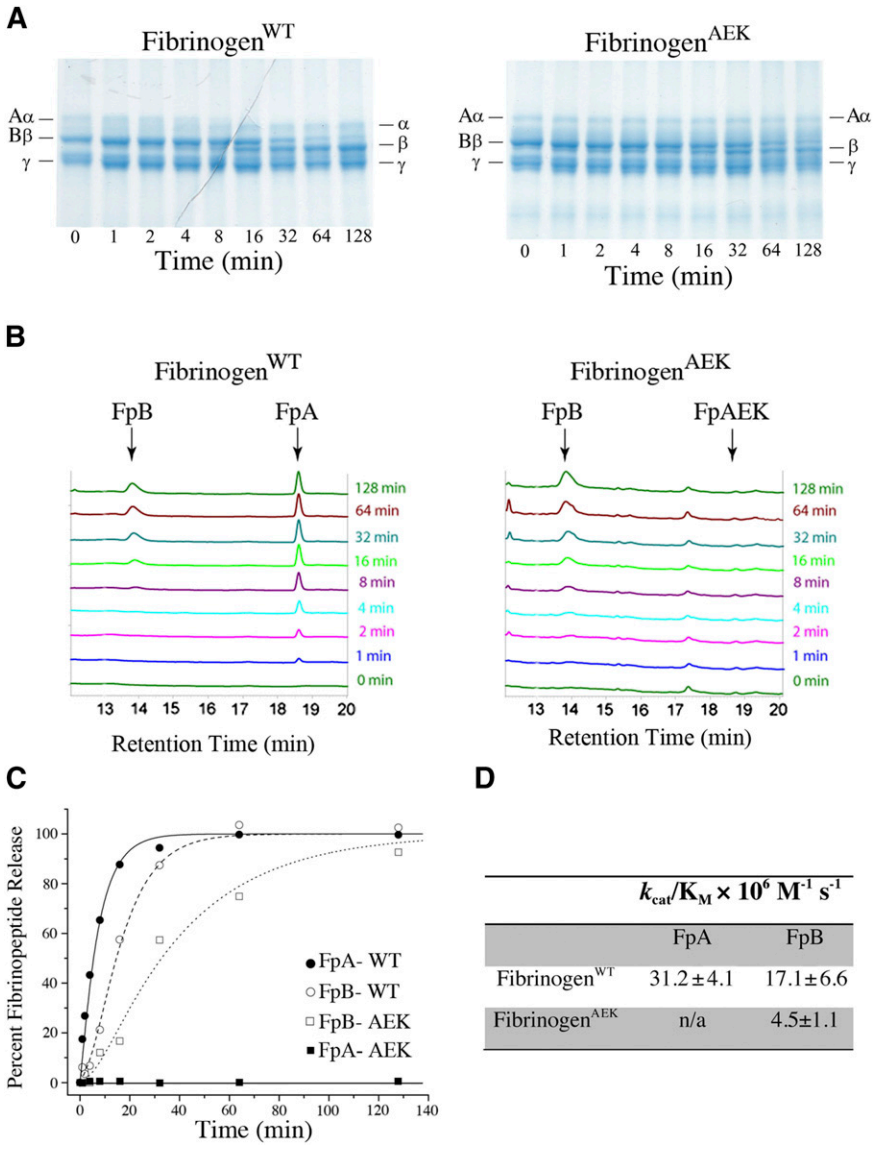

Figure 4. Failure of thrombin-mediated proteolytic release of the mutant fibrinopeptide A of fibrinogen isolated from Fib^{AEK} mice. (A) Reaction mixtures of purified WT fibrinogen (left) and fibrinogen^{AEK} (right) were incubated with thrombin and Ca^{2+} for various times, and the fibrinogen chains analyzed by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of the Aα, cleaved α, Bβ, cleaved β, and γ chains are indicated. Note that cleaved α chain was never observed in reaction mixtures containing fibrinogen^{AEK} regardless of incubation time. (B) HPLC chromatograms of reaction mixtures containing either WT fibrinogen (left) or fibrinogen^{AEK} (right) following incubation with thrombin and Ca^{2+} for various times. Indicated are the positions of peaks corresponding to fibrinopeptide A (FpA), fibrinopeptide B (FpB), and mutant fibrinopeptide A (FpAEK). The identification of peak positions was independently established by resolving synthetic peptides corresponding to the predicted fibrinopeptide sequence. Note that FpAEK is never observed in chromatograms derived from fibrinogen^{AEK} reactions regardless of incubation time with thrombin and Ca^{2+}. (C) Fibrinopeptide release curves were prepared by plotting the percent of fibrinopeptide released versus time. FpA data were fitted with a simple first-order equation, and the FpB data from normal fibrinogen were fitted to a standard equation describing 2 consecutive first-order processes. (D) Table of specificity constants (k_{cat}/K_M), which were determined by dividing first-order rate constants by the thrombin concentration.

capable of supporting *S aureus* bacterial clumping through the clumping factor A (ClfA) bacterial cell surface fibrinogen receptor (Figure 7E). *S aureus* produces coagulase (Coa), a virulence factor that is released into the extracellular milieu capable of binding prothrombin. Coa:prothrombin complexes mediate fibrin polymer formation independent of thrombin generation via the prothrombinase complex. Here, we found that culture supernatant obtained from Coa$^+$ *S aureus*, but not from Coa$^-$ *S aureus,* was capable of supporting fibrin polymer formation in plasma from WT mice (Figure 7F). In contrast, fibrin polymer formation was never detected in plasma from Fib^{AEK} following the addition of culture supernatant from either Coa$^+$ or Coa$^-$ *S aureus* (Figure 7F). Consistent with a significant failure of bacterial clearance, both Fib$^{-/-}$ and Fib^{AEK} mice displayed significantly worse survival profiles following intraperitoneal

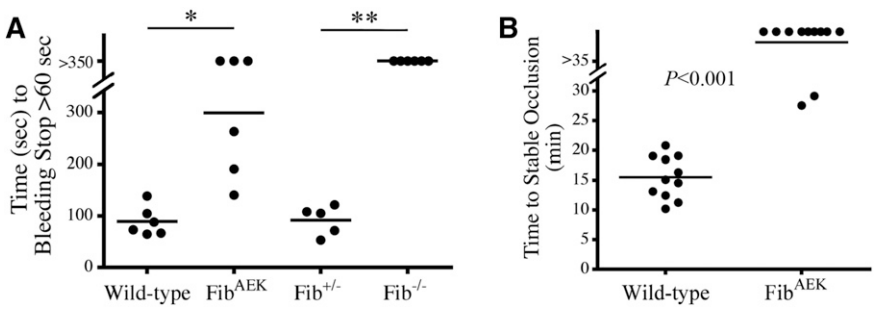

Figure 5. Fib^{AEK} mice display prolonged tail bleeding times and significant protection from occlusive thrombotic injury to the carotid artery. (A) Time to cessation of bleeding (>60 seconds) of WT, Fib^{AEK}, Fib$^{+/-}$, and Fib$^{-/-}$ mice following 3-mm excision of the distal portion of the tail. Horizontal bars indicate mean times for each group. Note that 3 of 6 Fib^{AEK} mice and 6 of 6 Fib$^{-/-}$ mice did not stop bleeding during the evaluation period. *$P < .01$ by Student *t* test; **$P < .01$ by Fisher's exact test. (B) Time to stable carotid vessel occlusion as indicated by detection of flow stop using a Doppler flowmeter following $FeCl_3$ injury. A statistically significant difference of $P < .001$ was determined by Fisher's exact test.

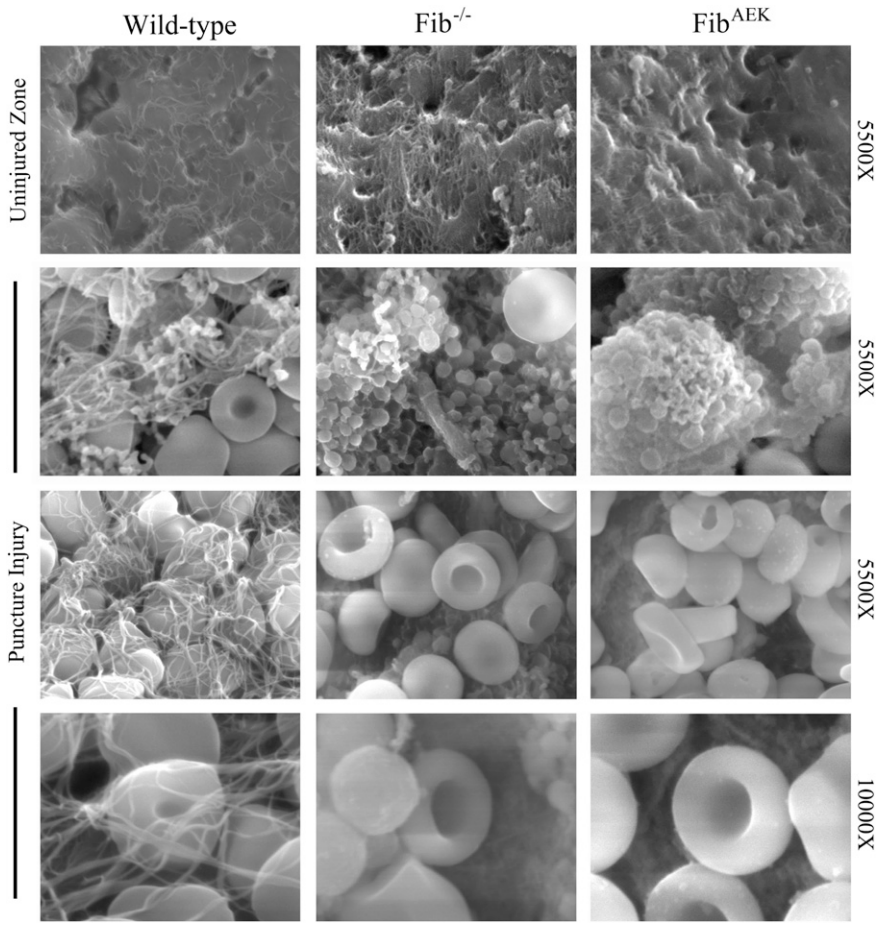

Figure 6. Failure of extravascular fibrin polymer formation in Fib^{AEK} mice following liver needle puncture injury. Representative scanning electron micrographs of right medial liver lobes from WT, Fib$^{-/-}$, and Fib^{AEK} mice from uninjured areas (top row) or of the injured zone following needle puncture injury (bottom 3 rows). Note that the injured zone of WT mice was characterized by fibrin polymer structures associated with platelets and RBCs. In contrast, the injured zones of livers from both Fib$^{-/-}$ and Fib^{AEK} mice displayed platelet-rich clusters and RBCs, but a distinct absence of fibrin polymer structures.

infection with 0.4×10^9 CFU *S aureus* relative to similarly challenged WT mice (Figure 7G). Intriguingly, when challenged with a higher dose of 1×10^9 CFU *S aureus*, Fib^{AEK} mice exhibited a far superior survival profile relative to Fib$^{-/-}$ mice challenged in parallel. Here, ~90% of Fib$^{-/-}$ mice succumbed to infection within 24 hours, whereas ~50% of Fib^{AEK} mice survived for the 2-week observation period (Figure 7H). Of note, none of the infected animals (including Fib$^{-/-}$ mice) displayed evidence of significant hemorrhage. Collectively, these findings suggest that in specific contexts soluble fibrinogen has important functional significance even beyond a contribution to hemostasis.

Discussion

Fibrin(ogen) exists as either a soluble monomer or as an insoluble polymer. Each of these dramatically different structural forms appears to have distinct properties thought to contribute to the full spectrum of physiologic and pathologic activities attributable to fibrin(ogen) in vivo. However, formally resolving the biologic contributions of each molecular form to reproductive success, hemostasis/thrombosis, tissue repair, disease, and inflammatory processes has remained technically unapproachable using available genetic and pharmacologic tools. The inability of investigators to formally distinguish the contributions of fibrinogen and fibrin in vivo has led to a half-century-old practice of using parentheses when referring to "fibrin(ogen)"-dependent events. The genetic alteration imposed here was guided by prior studies suggesting that eliminating FpA release alone would be sufficient to prevent thrombin-mediated fibrin polymer formation. The human Aα chain variants fibrinogen Metz and fibrinogen Frankfurt XIII have a mutation in the P1 residue (ie, Aα^{Arg16Cys}) that renders the Aα chain completely insensitive to thrombin cleavage and, comparable to our findings with fibrinogen^{AEK}, impeded clotting function.[29-32] Although fundamentally lacking the capacity to generate fibrin matrices in vivo, all other molecular/functional elements of the fibrinogen^{AEK} molecule and Fib^{AEK} mice remain intact, including (1) support of platelet aggregation/thrombus formation through the platelet integrin receptor $\alpha_{IIb}\beta_3$, (2) the potential to engage all other integrin and nonintegrin receptors (eg, $\alpha_M\beta_2$, vascular cell adhesion molecule-1) as no receptor binding motifs were selectively mutated in fibrinogen^{AEK}, (3) associations with circulating enzymes and proteins (eg, fXIII, fibronectin), (4) thrombin generation and thrombin action on all non-fibrinogen substrates (eg, fXIII, protein C and PARs), and (5) normal hemostatic factor levels. The availability of fibrinogen, either in a soluble form or immobilized form on other cell surface receptors or foreign bodies, may present biologic benefits never previously appreciated and impossible to recognize through comparative study of WT and fibrinogen-deficient mice. Thus, Fib^{AEK} mice provide a unique, clean experimental system for defining the biologic contribution(s) of fibrinogen monomer in any context in vivo that does not require other additional investigator-imposed manipulations such as fibrinogen- or other coagulation-targeted pharmacologic agents.

Hemostatic capacity in Fib^{AEK} mice was superior to that of Fib$^{-/-}$ mice, despite the lack of clotting function. Fib$^{-/-}$ mice consistently fail to stop blood flow following tail tip excision, whereas a significant

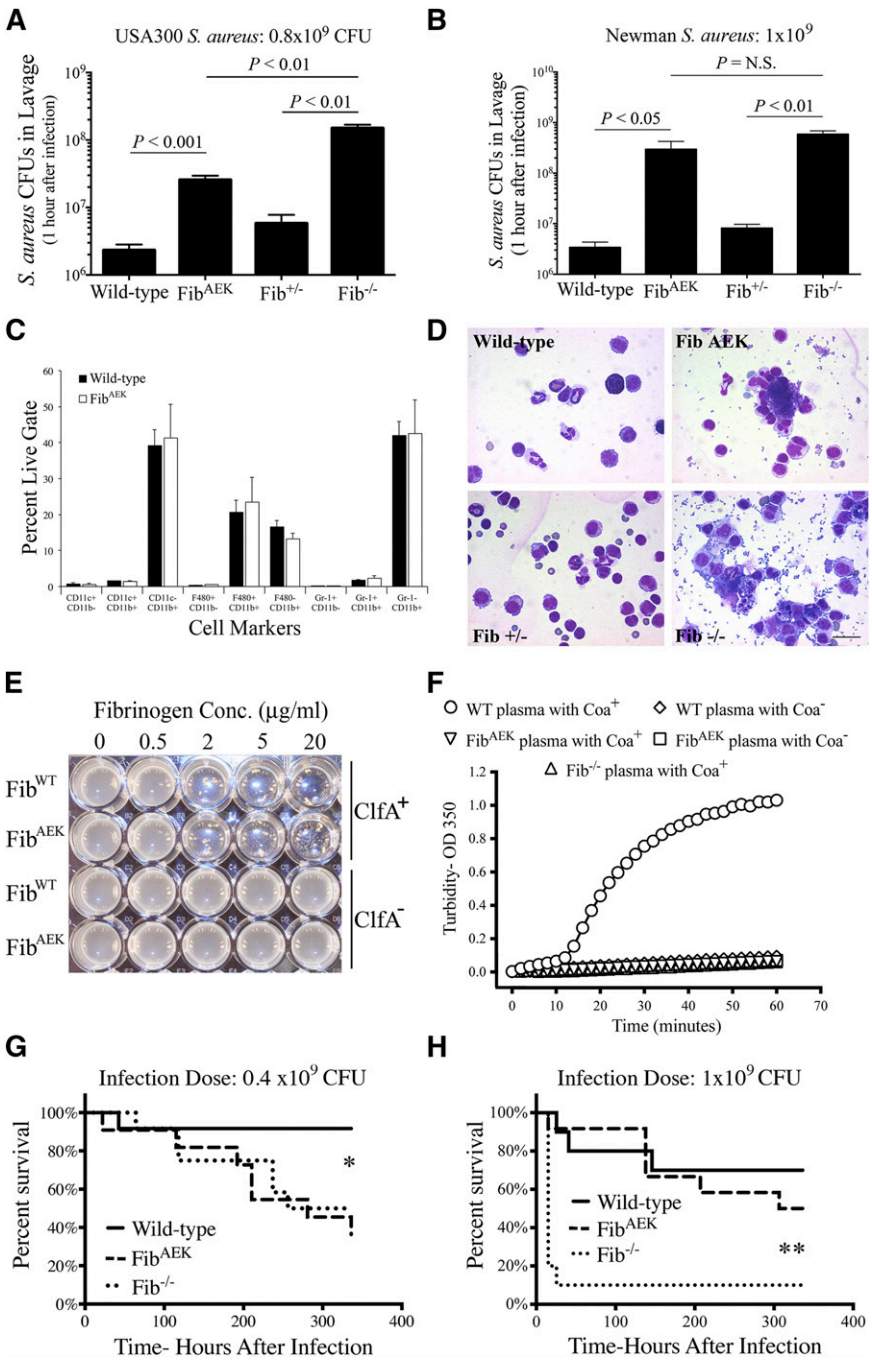

Figure 7. Fibrinogen^{AEK} does not support rapid clearance of *S aureus* bacteria following acute peritoneal infection but retains some capacity to limit host lethality. The number of *S aureus* CFUs present in peritoneal lavage fluid collected 1 hour after intraperitoneal infection with (A) 0.8 × 10^9 CFUs strain USA300 *S aureus* and (B) 1 × 10^9 CFUs strain Newman *S aureus* from WT, Fib^{AEK}, Fib$^{+/-}$, and Fib$^{-/-}$ mice (n = 6 per genotype). Data are presented as mean ± standard error of the mean with statistical comparisons made by Student *t* test. (C) Flow cytometric analysis of myeloid cell populations harvested from the peritoneal cavity of WT and Fib^{AEK} mice (n = 6 mice per genotype). Analyses are presented as the mean ± standard error of the mean. (D) Representative photomicrographs of cytospin preparations of peritoneal lavage fluid taken 1 hour after intraperitoneal infection with 1 × 10^9 CFUs *S aureus*. Note the cell-associated and free bacteria in samples from Fib^{AEK} and Fib$^{-/-}$ mice that are largely absent in samples from WT and Fib$^{+/-}$ mice. Scale bar, 20 μm. (E) Analysis of fibrinogen-dependent bacterial clumping with suspensions of strain Newman *S aureus* with and without expression of clumping factor A (*ClfA*). Notably, both WT and fibrinogen^{AEK} support bacterial clumping at a concentration of 2 μg/mL and above. (F) Analyses of coagulase (Coa)-induced plasma clotting with citrate-plasma preparations from WT, homozygous Fib^{AEK}, and Fib$^{-/-}$ mice using supernatants prepared from stationary phase cultures of both strain Newman WT (Coa$^+$) *S aureus* and Coa-negative (Coa$^-$) strain Newman *S aureus*. Survival analyses of WT, Fib^{AEK}, and Fib$^{-/-}$ mice (n = 12 per genotype) following intraperitoneal infection with (G) 0.4 × 10^9 CFUs or (H) 1 × 10^9 CFUs strain Newman *S aureus*. **P* < .01 for WT compared with Fib$^{-/-}$ or Fib^{AEK}; ***P* < .01 for Fib^{AEK} compared with Fib$^{-/-}$ using Kaplan-Meier log-rank analysis.

fraction of Fib^{AEK} animals ultimately control blood loss following this hemostatic challenge. Additionally, Fib^{AEK} mice exhibit superior perinatal survival relative to Fib$^{-/-}$ mice, a time when both genotypes are at high risk of spontaneous bleeding events. Because fibrinogen-dependent platelet aggregation is maintained in Fib^{AEK} mice, we postulate the enhanced hemostasis in Fib^{AEK} mice is, in part, the result of retained fibrinogen-platelet interactions. The requirement of fibrin polymer formation for hemostasis is likely a function of the severity of the hemostatic challenge, where fibrin clotting is mandatory for control of blood loss following severe vessel injuries. More formal analyses on the role of fibrin formation in a range of hemostatic challenges will be the focus of future studies with Fib^{AEK} mice. The Fib^{AEK} mutant also provides a means to further define the contributions of soluble fibrinogen vs fibrin in thrombus formation and the functional properties of distinct domains recognized within arterial thrombi. Following vascular injury, thrombi form with distinct structural features, including a fibrin-rich extravascular/perivascular zone, an intravascular/lumenal inner core of densely packed platelets on a fibrin base, and an outer shell composed of loosely packed platelets.[33,34] Our findings suggest soluble fibrinogen can contribute to thrombus formation, but fibrin polymer formation is required for the development of a fully occlusive thrombus following $FeCl_3$ injury of the carotid artery. Fibrin(ogen) has been documented within defined domains of the thrombus, but the precise requirements of fibrin polymer compared with soluble fibrinogen for thrombus architecture, growth, capping, and resolution, as well as dynamic changes within the thrombus (eg, solute transport within and between domains), are open questions now approachable for the first time with Fib^{AEK} mice.[35-37]

The present studies provide strong direct evidence that FpA release is critical to, and sufficient for, fibrin polymer formation. Both turbidity measurements and scanning electron microscopy studies revealed robust polymerization of fibrinogen^{AEK} following incubation with EK enzyme (supporting FpA, but not FpB, release), but a fundamental failure of polymerization following incubation with thrombin (supporting FpB, but not FpA, release). Our findings are compatible with prior biochemical analyses using snake venom proteases favoring FpA or FpB release,[27,38-41] as well as previous reports indicating that polymerization is compromised in the fibrinogen variant $\gamma^{Asp364His}$, which alters the "a" binding pocket, whereas polymerization is similar to normal in the fibrinogen variant $B\beta^{Arg432Ala}$, which disrupts the "b" binding pocket.[41-44] B:b interactions are reported to be exceptionally weak as characterized by high affinity constants and a low strength force to rupture the bonds.[43,45] Prior studies suggest any assembly based on cleavage of FpB alone would be restricted to nonphysiologic conditions of low salt concentrations and low temperatures.[29,39,46] Fib^{AEK} mice and fibrinogen^{AEK} derived from these animals provide novel tools and reagents for more comprehensive studies exploring the consequences of FpA release, FpB release, or both to polymer formation and clot structure both in vitro and in vivo. Intriguingly, over very long incubation times and under nonphysiologic conditions, fXIII transglutaminase is known to covalently join intact soluble fibrinogen into macromolecular assemblies in vitro in the absence of any kind of fibrinopeptide release.[47-50] Trace and disorganized macromolecular aggregates were occasionally encountered in scanning electron microscopy studies of reaction mixtures with fibrinogen^{AEK} and thrombin, potentially a reflection of fXIII activity in vitro. Thus, although no appreciable fibrin polymer formation could be detected in Fib^{AEK} mice, it remains to be established whether fXIII-mediated cross-linking of fibrinogen^{AEK} monomers occurs in vivo under any physiologic or pathologic conditions.

Fibrinogen is an important nexus of host-pathogen interaction as evidenced by the fact that many microbial pathogens have evolved and maintained bacterial-encoded fibrinogen binding proteins, direct plasminogen activators, and in the case of *S aureus*, 2 direct prothrombin activators: coagulase and vWbp.[51-55] Interestingly, in the setting of intravenous *S aureus* infection (bacteremia), fibrinogen supports pathogen virulence and investigator-imposed fibrinogen deficiency improves host survival,[14] whereas in the setting of *S aureus* peritonitis, fibrinogen diminishes pathogen virulence and investigator-imposed fibrinogen deficiency impedes bacterial clearance and reduces host survival.[15] An impediment in the clearance of *S aureus* in the peritoneal cavity was also documented for mice carrying a mutant form of fibrinogen lacking the $\alpha_M\beta_2$ binding motif but maintaining full clotting function.[16] As with Fib^{AEK} mice, elimination of the fibrinogen $\alpha_M\beta_2$-binding motif in Fib$\gamma^{390-396A}$ mice did not alter the composition or number of resident peritoneal immune cells. Retrieval of bacteria from the peritoneal cavity of Fib$\gamma^{390-396A}$ mice 1 hour after infection resulted in a 10-fold increase in CFUs relative to WT animals. These findings suggest both the importance of the fibrinogen $\alpha_M\beta_2$-binding motif in host defense and that fibrin formation is, in itself, insufficient to support the implementation of full antimicrobial function. Here, we show that fibrin polymer formation is vital to *S aureus* clearance and ultimately host survival in *S aureus*-induced peritonitis as we document an ~200-fold increase in CFUs retrieved from Fib^{AEK} relative to WT mice 1 hour after peritoneal infection. This finding is also compatible with the view that soluble fibrinogen is a relatively poor ligand for $\alpha_M\beta_2$, whereas fibrin polymer (or immobilized fibrinogen) is a strong ligand for this leukocyte integrin receptor.[7,56]

The finding that Fib^{AEK} animals displayed a survival advantage over Fib$^{-/-}$ mice following high-dose intraperitoneal *S aureus* infection provided evidence that soluble fibrinogen is also biologically meaningful to the host regarding infection outcome. The precise mechanism(s) remain(s) to be fully explored, but 2 general hypotheses stand out. First, *S aureus* engagement of soluble fibrinogen in Fib^{AEK} mice by way of known microbial fibrinogen binding proteins (eg, ClfA) may change infection outcome at high intraperitoneal *S aureus* loads by promoting bacterial aggregation and impeding dissemination out of the peritoneal cavity to distant organs.[53,57] Second, soluble fibrinogen interactions with host cells including platelets and inflammatory cells (ie, neutrophils and macrophages) may increase tolerance at high bacterial loads by supporting fibrinogen-mediated platelet interactions and platelet-linked bacterial killing mechanisms.[58-60] Alternatively, fibrinogen either in the soluble form or adhered to peritoneal surfaces may support inflammatory cell survival and antimicrobial function including the formation of neutrophil extracellular traps.[16,61-63] Whatever the precise mechanism(s) by which soluble fibrinogen limits host mortality relative to fibrinogen-deficient mice in this context, it does not appear to be coupled to overt hemorrhage. Furthermore, since fibrin(ogen) engagement of *S aureus* through ClfA is known to promote pathogen virulence and abscess formation following intravenous infection,[51,64,65] the advantage to the host conferred by soluble fibrinogen in Fib^{AEK} mice over Fib$^{-/-}$ mice in high-load *S aureus* peritonitis could be reversed to a disadvantage for the host following intravenous-induced *S aureus* bacteremia. Insights gained through more comprehensive studies of Fib^{AEK} mice will not only provide a better understanding of biologic processes linked to fibrinogen but also may guide the development of novel intervention strategies for a wide spectrum of diseases.

Acknowledgments

The authors thank Carolina Cruz, Cheryl Rewerts, Leah Rosenfeldt, Maureen Shaw, and Kathryn McElhinney for excellent technical assistance and Dr James Luyendyk and Dr John Weisel for helpful technical suggestions in the preparation of this manuscript.

This work was supported by National Institutes of Health grants from the National Heart, Lung, and Blood Institute HL096126 (J.L.D.), from the National Institute of Arthritis and Musculoskeletal and Skin Diseases AR056990 (M.J.F.), and Cincinnati Rheumatic Diseases Center-Animal Models of Inflammatory Diseases core grant from the National Institute of Arthritis and Musculoskeletal and Skin Diseases P30 AR47363 (M.J.F.).

Authorship

Contribution: J.M.P., O.V.G., S.T., T.D., S.R.C., J.L.D., and M.J.F. designed the research, performed experiments, analyzed the data, and wrote the manuscript; Y.-P.K., M.H., E.S.M., and J.S.P. provided critical guidance on experimental procedures and helped write the manuscript; and all authors read and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Matthew J. Flick, Cincinnati Children's Hospital Research Foundation, Division of Experimental Hematology and Cancer Biology, ML7015, 3333 Burnet Ave, Cincinnati, OH 45229-3039; e-mail: matthew.flick@cchmc.org.

References

1. Blokhin IO, Lentz SR. Mechanisms of thrombosis in obesity. *Curr Opin Hematol.* 2013;20(5): 437-444.
2. Esmon CT. Crosstalk between inflammation and thrombosis. *Maturitas.* 2004;47(4):305-314.
3. Palumbo JS. Mechanisms linking tumor cell-associated procoagulant function to tumor dissemination. *Semin Thromb Hemost.* 2008; 34(2):154-160.
4. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. *Semin Immunopathol.* 2012;34(1):43-62.
5. Katagiri Y, Hiroyama T, Akamatsu N, Suzuki H, Yamazaki H, Tanoue K. Involvement of alpha v beta 3 integrin in mediating fibrin gel retraction. *J Biol Chem.* 1995;270(4):1785-1790.
6. Suehiro K, Gailit J, Plow EF. Fibrinogen is a ligand for integrin alpha5beta1 on endothelial cells. *J Biol Chem.* 1997;272(8):5360-5366.
7. Ugarova TP, Solovjov DA, Zhang L, et al. Identification of a novel recognition sequence for integrin alphaM beta2 within the gamma-chain of fibrinogen. *J Biol Chem.* 1998;273(35): 22519-22527.
8. Bach TL, Barsigian C, Yaen CH, Martinez J. Endothelial cell VE-cadherin functions as a receptor for the beta15-42 sequence of fibrin. *J Biol Chem.* 1998;273(46):30719-30728.
9. Languino LR, Duperray A, Joganic KJ, Fornaro M, Thornton GB, Altieri DC. Regulation of leukocyte-endothelium interaction and leukocyte transendothelial migration by intercellular adhesion molecule 1-fibrinogen recognition. *Proc Natl Acad Sci USA.* 1995; 92(5):1505-1509.
10. Altieri DC. Regulation of leukocyte-endothelium interaction by fibrinogen. *Thromb Haemost.* 1999; 82(2):781-786.
11. Rubel C, Fernández GC, Dran G, Bompadre MB, Isturiz MA, Palermo MS. Fibrinogen promotes neutrophil activation and delays apoptosis. *J Immunol.* 2001;166(3):2002-2010.
12. Rubel C, Fernández GC, Rosa FA, et al. Soluble fibrinogen modulates neutrophil functionality through the activation of an extracellular signal-regulated kinase-dependent pathway. *J Immunol.* 2002;168(7):3527-3535.
13. Rubel C, Gómez S, Fernández GC, Isturiz MA, Caamaño J, Palermo MS. Fibrinogen-CD11b/CD18 interaction activates the NF-kappa B pathway and delays apoptosis in human neutrophils. *Eur J Immunol.* 2003;33(5): 1429-1438.
14. Flick MJ, Du X, Prasad JM, et al. Genetic elimination of the binding motif on fibrinogen for the S. aureus virulence factor ClfA improves host survival in septicemia. *Blood.* 2013;121(10): 1783-1794.
15. Mullarky IK, Szaba FM, Berggren KN, et al. Infection-stimulated fibrin deposition controls hemorrhage and limits hepatic bacterial growth during listeriosis. *Infect Immun.* 2005;73(7): 3888-3895.
16. Flick MJ, Du X, Witte DP, et al. Leukocyte engagement of fibrin(ogen) via the integrin receptor alphaMbeta2/Mac-1 is critical for host inflammatory response in vivo. *J Clin Invest.* 2004;113(11):1596-1606.
17. Vanassche T, Verhaegen J, Peetermans WE, et al. Inhibition of staphylothrombin by dabigatran reduces Staphylococcus aureus virulence. *J Thromb Haemost.* 2011;9(12):2436-2446.
18. Mullins ES, Kombrinck KW, Talmage KE, et al. Genetic elimination of prothrombin in adult mice is not compatible with survival and results in spontaneous hemorrhagic events in both heart and brain. *Blood.* 2009;113(3):696-704.
19. Light A, Janska H. Enterokinase (enteropeptidase): comparative aspects. *Trends Biochem Sci.* 1989;14(3):110-112.
20. Yuan X, Zheng X, Lu D, Rubin DC, Pung CY, Sadler JE. Structure of murine enterokinase (enteropeptidase) and expression in small intestine during development. *Am J Physiol.* 1998; 274(2 Pt 1):G342-G349.
21. Suh TT, Holmbäck K, Jensen NJ, et al. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes Dev.* 1995;9(16):2020-2033.
22. Sun WY, Witte DP, Degen JL, et al. Prothrombin deficiency results in embryonic and neonatal lethality in mice. *Proc Natl Acad Sci USA.* 1998; 95(13):7597-7602.
23. Xue J, Wu Q, Westfield LA, et al. Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. *Proc Natl Acad Sci USA.* 1998;95(13):7603-7607.
24. Galanakis DK, Henschen A, Peerschke EI, Kehl M. Fibrinogen Stony Brook, a heterozygous A alpha 16Arg——Cys dysfibrinogenemia. Evaluation of diminished platelet aggregation support and of enhanced inhibition of fibrin assembly. *J Clin Invest.* 1989;84(1):295-304.
25. Lewis SD, Shields PP, Shafer JA. Characterization of the kinetic pathway for liberation of fibrinopeptides during assembly of fibrin. *J Biol Chem.* 1985;260(18):10192-10199.
26. Ruf W, Bender A, Lane DA, Preissner KT, Selmayr E, Müller-Berghaus G. Thrombin-induced fibrinopeptide B release from normal and variant fibrinogens: influence of inhibitors of fibrin polymerization. *Biochim Biophys Acta.* 1988; 965(2-3):169-175.
27. Okumura N, Terasawa F, Haneishi A, et al. B:b interactions are essential for polymerization of variant fibrinogens with impaired holes 'a'. *J Thromb Haemost.* 2007;5(12):2352-2359.
28. Pechik I, Yakovlev S, Mosesson MW, Gilliland GL, Medved L. Structural basis for sequential cleavage of fibrinopeptides upon fibrin assembly. *Biochemistry.* 2006;45(11):3588-3597.
29. Mosesson MW, DiOrio JP, Müller MF, et al. Studies on the ultrastructure of fibrin lacking fibrinopeptide B (beta-fibrin). *Blood.* 1987;69(4): 1073-1081.
30. Soria J, Soria C, Boulard C. [Structural anomaly of Metz fibrinogen, localized on the (A) chain of the molecule]. *Biochimie.* 1972;54(3):415-417.
31. Soria J, Soria C, Samama M, Poirot E, Kling C. Fibrinogen Troyes–fibrinogen Metz. Two new cases of congenital dysfibrinogenemia. *Thromb Diath Haemorrh.* 1972;27(3):619-633.
32. Galanakis D, Spitzer S, Scharrer I. Unusual A alpha 16Arg–>Cys dysfibrinogenaemic family: absence of normal A alpha-chains in fibrinogen from two of four heterozygous siblings. *Blood Coagul Fibrinolysis.* 1993;4(1):67-71.
33. Stalker TJ, Traxler EA, Wu J, et al. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. *Blood.* 2013;121(10):1875-1885.
34. Kamocka MM, Mu J, Liu X, et al. Two-photon intravital imaging of thrombus development. *J Biomed Opt.* 2010;15(1):016020.
35. Stalker TJ, Welsh JD, Tomaiuolo M, et al. A systems approach to hemostasis: 3. Thrombus consolidation regulates intrathrombus solute transport and local thrombin activity. *Blood.* 2014; 124(11):1824-1831.
36. Tomaiuolo M, Stalker TJ, Welsh JD, Diamond SL, Sinno T, Brass LF. A systems approach to hemostasis: 2. Computational analysis of molecular transport in the thrombus microenvironment. *Blood.* 2014;124(11): 1816-1823.
37. Welsh JD, Stalker TJ, Voronov R, et al. A systems approach to hemostasis: 1. The interdependence of thrombus architecture and agonist movements in the gaps between platelets. *Blood.* 2014; 124(11):1808-1815.
38. Weisel JW. Fibrin assembly. Lateral aggregation and the role of the two pairs of fibrinopeptides. *Biophys J.* 1986;50(6):1079-1093.
39. Shainoff JR, Dardik BN. Fibrinopeptide B and aggregation of fibrinogen. *Science.* 1979; 204(4389):200-202.
40. Herzig RH, Ratnoff OD, Shainoff JR. Studies on a procoagulant fraction of southern copperhead snake venom: the preferential release of fibrinopeptide B. *J Lab Clin Med.* 1970;76(3): 451-465.
41. Soya K, Terasawa F, Okumura N. Fibrinopeptide A release is necessary for effective B:b interactions in polymerisation of variant fibrinogens with impaired A:a interactions. *Thromb Haemost.* 2013;109(2):221-228.
42. Okumura N, Gorkun OV, Lord ST. Severely impaired polymerization of recombinant fibrinogen gamma-364 Asp–> His, the substitution discovered in a heterozygous individual. *J Biol Chem.* 1997;272(47): 29596-29601.
43. Litvinov RI, Gorkun OV, Galanakis DK, et al. Polymerization of fibrin: Direct observation and quantification of individual B:b knob-hole interactions. *Blood.* 2007;109(1):130-138.
44. Bowley SR, Lord ST. Fibrinogen variant BbetaD432A has normal polymerization but does not bind knob "B". *Blood.* 2009;113(18): 4425-4430.
45. Geer CB, Tripathy A, Schoenfisch MH, Lord ST, Gorkun OV. Role of 'B-b' knob-hole interactions in fibrin binding to adsorbed fibrinogen. *J Thromb Haemost.* 2007;5(12):2344-2351.
46. Shainoff JR, Dardik BN. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. *Ann N Y Acad Sci.* 1983;408:254-268.
47. Blombäck B, Banerjee D, Carlsson K, et al. Native fibrin gel networks and factors influencing their formation in health and disease. *Adv Exp Med Biol.* 1990;281:1-23.
48. Blombäck B, Procyk R, Adamson L, Hessel B. FXIII induced gelation of human fibrinogen–an alternative thiol enhanced, thrombin independent pathway. *Thromb Res.* 1985;37(5): 613-627.
49. Procyk R, Adamson L, Block M, Blombäck B. Factor XIII catalyzed formation of fibrinogen-fibronectin oligomers–a thiol enhanced process. *Thromb Res.* 1985;40(6):833-852.
50. Siebenlist KR, Meh DA, Mosesson MW. Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin. *Thromb Haemost.* 2001;86(5):1221-1228.
51. Cheng AG, McAdow M, Kim HK, Bae T, Missiakas DM, Schneewind O. Contribution of coagulases towards Staphylococcus aureus disease and protective immunity. *PLoS Pathog.* 2010;6(8): e1001036.
52. Foster TJ, Höök M. Surface protein adhesins of Staphylococcus aureus. *Trends Microbiol.* 1998; 6(12):484-488.
53. McDevitt D, Nanavaty T, House-Pompeo K, et al. Characterization of the interaction between

the Staphylococcus aureus clumping factor (ClfA) and fibrinogen. *Eur J Biochem.* 1997; 247(1):416-424.

54. Panizzi P, Nahrendorf M, Figueiredo JL, et al. In vivo detection of Staphylococcus aureus endocarditis by targeting pathogen-specific prothrombin activation. *Nat Med.* 2011;17(9): 1142-1146.
55. Geoghegan JA, Ganesh VK, Smeds E, Liang X, Höök M, Foster TJ. Molecular characterization of the interaction of staphylococcal microbial surface components recognizing adhesive matrix molecules (MSCRAMM) ClfA and Fbl with fibrinogen. *J Biol Chem.* 2010;285(9):6208-6216.
56. Lishko VK, Kudryk B, Yakubenko VP, Yee VC, Ugarova TP. Regulated unmasking of the cryptic binding site for integrin alpha M beta 2 in the gamma C-domain of fibrinogen. *Biochemistry.* 2002;41(43):12942-12951.
57. Kapral FA. Clumping of Staphylococcus aureus in the peritoneal cavity of mice. *J Bacteriol.* 1966; 92(4):1188-1195.
58. Yeaman MR. The role of platelets in antimicrobial host defense. *Clin Infect Dis.* 1997;25(5):951-968, quiz 969-970.
59. Yeaman MR. Platelets in defense against bacterial pathogens. *Cell Mol Life Sci.* 2010;67(4): 525-544.
60. Zhang X, Liu Y, Gao Y, et al. Inhibiting platelets aggregation could aggravate the acute infection caused by Staphylococcus aureus. *Platelets.* 2011;22(3):228-236.
61. Caudrillier A, Kessenbrock K, Gilliss BM, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest.* 2012;122(7):2661-2671.
62. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science.* 2004;303(5663):1532-1535.
63. Pluskota E, Soloviev DA, Szpak D, Weber C, Plow EF. Neutrophil apoptosis: selective regulation by different ligands of integrin alphaMbeta2. *J Immunol.* 2008;181(5): 3609-3619.
64. Cheng AG, Kim HK, Burts ML, Krausz T, Schneewind O, Missiakas DM. Genetic requirements for Staphylococcus aureus abscess formation and persistence in host tissues. *FASEB J.* 2009;23(10):3393-3404.
65. Guggenberger C, Wolz C, Morrissey JA, Heesemann J. Two distinct coagulase-dependent barriers protect Staphylococcus aureus from neutrophils in a three dimensional in vitro infection model. *PLoS Pathog.* 2012;8(1):e1002434.