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

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

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

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, FibAEK 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 FibAEK mice developed to term and exhibited postnatal survival superior to that of fibrinogen-deficient mice. Unlike fibrinogen-deficient mice, platelet-rich plasma from FibAEK mice supported normal platelet aggregation in vitro, highlighting that fibrinogenAEK retains the functional capacity to support interactions with platelets. Thrombin failed to release fibrinopeptide-A from fibrinogenAEK and failed to induce polymer formation with FibAEK plasma or purified fibrinogenAEK in 37°C mixtures regardless of incubation time. FibAEK 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 FeCl3 injury of carotid arteries. FibAEK mice exhibited a profound impediment in following liver injury, as assessed by electron microscopy, and a failure to generate stable occlusive thrombi following FeCl3 injury of carotid arteries. FibAEK 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 FeCl3 injury of carotid arteries.

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 exub tant or persistent fibrin(ogen) is associated with many diseases, including cancer, vessel wall disease, and inflammatory diseases. 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 aIIBb3-mediated platelet aggregation/thrombus formation. Similarly, leukocyte engagement of immobilized fibrinogen in vitro through integrin aIIb and nonintegrin receptors is thought to support cell adhesion, migration, phagocytosis, nuclear factor-kB–mediated transcription, chemokine and cytokine elaboration, degranulation, and other processes. 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, fibrinogen 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. 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. Similar studies using mice with a genetically imposed reduction in circulating prothrombin or pharmacologic inhibition of thrombin activity also resulted in...
significantly compromised *S. aureus* clearance from the peritoneal cavity. 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 fibrinogen 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 FibAEK 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 FibAEK mice to resolve whether fibrinogen or fibrin is the molecular form of host fibrinogen central to biologic outcome is exemplified through studies of fibrinogen-mediated host defense in *S. aureus* peritonitis.

### Materials and methods

#### Generation of FibAEK gene-targeted mice
Details of gene targeting in mouse embryonic stem cells and of the generation of FibAEK mice can be found in the supplemental Materials and Methods available on the Blood Web site.

#### Analysis of FibAEK 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 intravitral microscopy
FeCl3 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.

#### CIFA-dependent *S. aureus* aggregation and coagulase-induced plasma clots
The capacity of purified fibrinogenAEK to support bacterial aggregation in vitro with wild-type *S. aureus* and participate in coagulase-induced polymerization was established 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 E9GGGVRP1 were converted to A9DDDRP1 (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. The choice of generating an EK cleavage site was based on the unusual sequence specificity of EK (P4-P1 residues DDDD), 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 DDDD sequences in the 3 fibrinogen chains, and the fact that endogenous EK is a membrane-associated serine protease expressed only in the duodenum, 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 FibAEK and wild-type (WT) mice.

#### FibAEK blood fails to support thrombin-induced clot formation but can support platelet aggregation in vitro

The capacity of thrombin to induce polymer formation for fibrinogenAEK 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 FibAEK 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 FibAEK mice failed to clot regardless of observation period at 37°C, whereas plasma from WT mice had average clottingtimes 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 FibAEK mice (Figure 2C). Even 18 hours after thromboplastin addition, neither clot formation nor retraction could be appreciated in FibAEK whole blood samples; the only observable event was red cell sedimentation. Nevertheless, the overall structural integrity of circulating fibrinogenAEK was strongly inferred by the capacity of FibAEK platelet-rich plasma, but not

###  *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.
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. However, homozygous Fib$^{AEK}$ offspring surviving the perinatal
period generally survived well into adulthood in the absence of other challenges. To formally compare the perinatal survival profile of homozygous Fib\textsuperscript{AEK} mice and homozygous fibrinogen-deficient (Fib\textsuperscript{−/−}) 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\textsuperscript{AEK} offspring survived to weaning relative to Fib\textsuperscript{−/−} offspring (Table 2).

Heterozygous Fib\textsuperscript{AEK} female mice consistently carried litters to term without hemorrhagic consequences, but homozygous Fib\textsuperscript{AEK} female mice were unable to sustain pregnancies. Ten of 10 homozygous Fib\textsuperscript{AEK} mice impregnated by Fib\textsuperscript{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\textsuperscript{AEK} mice revealed massive intrauterine hemorrhage characterized by free maternal blood cells (evidenced by enucleated red
blood cells) within the uterus and placental tissue (supplemental Figure 1). Notably, the developing homozygous Fib\textsuperscript{AEK} embryos appeared developmentally sound, with no evidence of free embryonic (nucleated) red blood cells (supplemental Figure 1).

**Fibrinogen\textsuperscript{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\textsuperscript{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\textsuperscript{AEK}, but not plasma from Fib\textsuperscript{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\textsuperscript{AEK} was similar to those generated with thrombin-cleaved WT fibrinogen, with the exception that the fibrin in the former seemed generally thicker (Figure 3E, far right and far left). Diffuse and thin macromolecular structures were observed in reactions of fibrinogen\textsuperscript{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\textsuperscript{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\textsuperscript{AEK} reactions regardless of incubation time with thrombin (Figure 4A). Even fibrinogen\textsuperscript{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\textsuperscript{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\textsuperscript{WT} and fibrinogen\textsuperscript{AEK} (Figure 4A-B). However, consistent with findings with other fibrinogen variants,\textsuperscript{24-28} HPLC analyses suggested that FpB release from fibrinogen\textsuperscript{AEK} was delayed relative to thrombin-mediated release of FpB from WT fibrinogen (Figure 4B-C). The calculated specificity constants (k\textsubscript{cat}/K\textsubscript{m}) confirmed a significant diminution in the efficiency of FpB release from fibrinogen\textsuperscript{AEK} relative to WT fibrinogen (Figure 4D).

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

To evaluate the ability of Fib\textsuperscript{AEK} mice to control blood loss following acute vessel injury, we compared tail-bleeding times in cohorts of WT, Fib\textsuperscript{AEK}, Fib\textsuperscript{−/−}, and Fib\textsuperscript{−/+} mice. As shown in Figure 5A, WT and Fib\textsuperscript{−/+} mice rapidly and uniformly stopped bleeding following tail tip amputation, whereas Fib\textsuperscript{AEK} and Fib\textsuperscript{−/−} mice exhibited a major, albeit not identical, impediment in the control of blood loss. Unlike Fib\textsuperscript{−/−} mice, which uniformly failed to stop blood loss over the entire observation period (>6 minutes), half of Fib\textsuperscript{AEK} mice analyzed ultimately stopped blood loss within this timeframe (Figure 5A). Thus, although hemostasis is compromised in Fib\textsuperscript{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\textsuperscript{AEK} mice were challenged with FeCl\textsubscript{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\textsuperscript{AEK} mice challenged never displayed complete vessel occlusion during the observation period (>35 minutes). The Doppler flow tracings for Fib\textsuperscript{AEK} mice suggested embolization events in all but 1 Fib\textsuperscript{AEK} animal (data not shown). Stable occlusion occurred in 2 Fib\textsuperscript{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\textsuperscript{AEK} in vitro results in compromised hemostasis and resistance to occlusive thrombus formation in vivo.

**Fib\textsuperscript{AEK} mice display a lack of extravascular fibrin deposition following liver injury**

To directly interrogate the capacity of Fib\textsuperscript{AEK} mice to generate extravascular fibrin polymer in an in vivo setting, we challenged WT, Fib\textsuperscript{−/−}, and Fib\textsuperscript{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 1. Hematologic profile of Fib\textsuperscript{AEK} mice**

<table>
<thead>
<tr>
<th></th>
<th>Fib\textsuperscript{WT} (N = 6)</th>
<th>Fib\textsuperscript{AEK} (N = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (&gt;10\textsuperscript{9}/L)</td>
<td>4.95 ± 1.7</td>
<td>4.79 ± 1.8</td>
</tr>
<tr>
<td>RBC (&gt;10\textsuperscript{12}/L)</td>
<td>8.92 ± 0.5</td>
<td>9.02 ± 0.5</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>12.15 ± 0.7</td>
<td>12.23 ± 0.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>51.52 ± 3.7</td>
<td>50.80 ± 2.9</td>
</tr>
<tr>
<td>Platelets (&gt;10\textsuperscript{11}/L)</td>
<td>1005 ± 157</td>
<td>982 ± 145</td>
</tr>
<tr>
<td>PT (seconds)</td>
<td>12.5 ± 0.2</td>
<td>&gt;180</td>
</tr>
<tr>
<td>aPTT (seconds)</td>
<td>29.3 ± 2.7</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Thrombin time (seconds)</td>
<td>14.9 ± 1.1</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

**Table 2. Analysis of postnatal survival comparing offspring with complete fibrinogen deficiency (Fib\textsuperscript{−/−}) and mice homozygous for Fib\textsuperscript{AEK}**

<table>
<thead>
<tr>
<th>Offspring genotype</th>
<th>Fib\textsuperscript{−/−} × Fib\textsuperscript{−/−}</th>
<th>Fib\textsuperscript{−/−} × Fib\textsuperscript{AEK}</th>
<th>Fib\textsuperscript{−/−} × Fib\textsuperscript{−/+}</th>
<th>Fib\textsuperscript{−/−} × Fib\textsuperscript{AEK}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mendelian ratio</td>
<td>1 1</td>
<td>1 1</td>
<td>1 1</td>
<td>1 1</td>
</tr>
<tr>
<td>No. observed (at 3 weeks)</td>
<td>170 85</td>
<td>130 93</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>% of expected*</td>
<td>100% 50%</td>
<td>100% 72%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < .05, \chi\textsuperscript{2} analysis.
of platelets associated with the polymer and other areas revealing primarily polymers rich in red blood cells (representative views in Figure 6, left). Fib<sup>−/−</sup> mice challenged in parallel displayed a total absence of polymer within the injured zones. Here, fields within the injured zone of Fib<sup>−/−</sup> livers were rich in platelet clusters or free red blood cells (representative views in Figure 6, center). Importantly, studies of Fib<sup>AEK</sup> 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<sup>AEK</sup> mice were essentially indistinguishable from those observed in Fib<sup>−/−</sup> mice (Figure 6), despite the fact that Fib<sup>AEK</sup> animals retain normal circulating levels of fibrinogen.

Fib<sup>AEK</sup> mice fail to efficiently clear <i>S. aureus</i> 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<sup>AEK</sup> mice, we sought to directly test the hypothesis that polymer formation is key to the implementation of the fibrinogen-dependent antimicrobial response in <i>S. aureus</i> peritonitis challenge. Multiple prior reports have indicated that fibrinogen is critical for a rapid and robust clearance of <i>S. aureus</i> from the peritoneal cavity following an intraperitoneal infection. However, the precise mechanism, and whether this host antimicrobial response was fibrinogen- or fibrin-dependent, has remained unknown. Consistent with previous reports, WT mice challenged with an intraperitoneal injection of ~10<sup>9</sup> colony-forming units (CFUs) of either the clinical isolate methicillin-resistant <i>S. aureus</i> (MRSA) strain USA300 (Figure 7A) or strain Newman WT <i>S. aureus</i> (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<sup>AEK</sup> mice and virtually the entire initial CFU inoculum of USA300 was retrieved from Fib<sup>−/−</sup> mice (Figure 7A). Similarly, the same numbers of strain Newman CFUs were retrieved by peritoneal lavage of Fib<sup>AEK</sup> and Fib<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>AEK</sup> 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<sup>AEK</sup> mice were indistinguishable. Cytospin preparations of lavage fluid from challenged Fib<sup>AEK</sup> mice and Fib<sup>−/−</sup> mice confirmed a persistence of large numbers of free bacteria in the peritoneal cavity of these animals, whereas preparations from WT or Fib<sup>−/−</sup> mice were largely devoid of bacteria (Figure 7D). In vitro analyses indicated that both WT fibrinogen and fibrinogen<sup>AEK</sup> were...
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 FibAEK 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
infection with $0.4 \times 10^9$ CFU *S. aureus* relative to similarly challenged WT mice (Figure 7G). Intriguingly, when challenged with a higher dose of $1 \times 10^9$ CFU *S. aureus*, Fib$^{AEK}$ mice exhibited a far superior survival profile relative to Fib$^{-/-}$ mice challenged in parallel. Here, $\sim 90\%$ of Fib$^{-/-}$ mice succumbed to infection within 24 hours, whereas $\sim 50\%$ 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 formation. The human Aα chain variants fibrinogen Metz and fibrinogen Frankfurt XIII have a mutation in the P1 residue (ie, AαArg16Cys) that renders the Aα chain completely insensitive to thrombin cleavage and, comparable to our findings with fibrinogen$^{AEK}$, impeded clotting function. 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, IXIII, fibronectin), (4) thrombin generation and thrombin action on all non-fibrinogen substrates (eg, IXIII, 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
Fraction of FibAEK animals ultimately control blood loss following this hemostatic challenge. Additionally, FibAEK mice exhibit superior perinatal survival relative to Fib\(^2/2\) mice, a time when both genotypes are at high risk of spontaneous bleeding events. Because fibrinogen-dependent platelet aggregation is maintained in FibAEK mice, we postulate the enhanced hemostasis in FibAEK 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 FibAEK mice. The FibAEK 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. 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 FibAEK mice.
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\textsuperscript{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 binding motif in Fib, as well as previous reports indicating that polymerization is compromised in the fibrinogen variant \(\gamma^{364HAs}\), which alters the “a” binding pocket, whereas polymerization is similar to normal in the fibrinogen variant \(\beta^2\text{Arg32Ala}\), which disrupts the “b” binding pocket.\textsuperscript{31-44} B:β interactions are reported to be exceptionally weak as characterized by high affinity constants and a low strength force to rupture the bonds.\textsuperscript{53,65} Prior studies suggest any assembly based on cleavage of FpB alone would be restricted to nonphysiologic conditions of low salt concentrations and low temperatures.\textsuperscript{29,39,46} Fib\textsuperscript{AEK} mice and fibrinogen\textsuperscript{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, F\textsubscript{XI}II transglutaminase is known to covalently join intact soluble fibrinogen into macromolecular assemblies in vitro in the absence of any kind of fibrinopeptide release.\textsuperscript{47-50} Trace and disorganized macromolecular aggregates were occasionally encountered in scanning electron microscopy studies of reaction mixtures with fibrinogen\textsuperscript{AEK} and thrombin, potentially a reflection of F\textsubscript{XIII} activity in vitro. Thus, although no appreciable fibrin polymer formation could be detected in Fib\textsuperscript{AEK} mice, it remains to be established whether F\textsubscript{XIII}-mediated cross-linking of fibrinogen\textsuperscript{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 \textit{S. aureus}, 2 direct prothrombin activators: coagulase and vWbp.\textsuperscript{51-55} Interestingly, in the setting of intravenous \textit{S. aureus} infection (bacteremia), fibrinogen supports pathogen virulence and investigator-imposed fibrinogen deficiency improves host survival,\textsuperscript{14} whereas in the setting of \textit{S. aureus} peritonitis, fibrinogen diminishes pathogen virulence and investigator-imposed fibrinogen deficiency impedes bacterial clearance and reduces host survival.\textsuperscript{15} An impediment in the clearance of \textit{S. aureus} in the peritoneal cavity was also documented for mice carrying a mutant form of \(\alpha\textsubscript{2}\text{M}\textsubscript{B}\textsubscript{2}\), binding motif but maintaining full clotting function.\textsuperscript{16} As with Fib\textsuperscript{−/−} mice, elimination of the fibrinogen \(\alpha\textsubscript{2}\text{M}\textsubscript{B}\textsubscript{2}\) binding motif in Fib\textsuperscript{390-396A} mice did not alter the composition or number of resident peritoneal immune cells. Retrieval of bacteria from the peritoneal cavity of Fib\textsuperscript{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 \(\alpha\textsubscript{2}\text{M}\textsubscript{B}\textsubscript{2}\) 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 \textit{S. aureus} clearance and ultimately host survival in \textit{S. aureus}-induced peritonitis as we document an \(\sim 200\)-fold increase in CFUs retrieved from Fib\textsuperscript{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 \(\alpha\textsubscript{2}\text{M}\textsubscript{B}\textsubscript{2}\), whereas fibrin polymer (or immobilized fibrinogen) is a strong ligand for this leukocyte integrin receptor.\textsuperscript{7,56}

The finding that Fib\textsuperscript{AEK} animals displayed a survival advantage over Fib\textsuperscript{−/−} mice following high-dose intraperitoneal \textit{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, \textit{S. aureus} engagement of soluble fibrinogen in Fib\textsuperscript{AEK} mice by way of known microbial fibrinogen binding proteins (eg, CIFA) may change infection outcome at high intraperitoneal \textit{S. aureus} loads by promoting bacterial aggregation and impeding dissemination out of the peritoneal cavity to distant organs.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \textit{S. aureus} through CIFA is known to promote pathogen virulence and abscissa formation following intravenous infection,\textsuperscript{51,64,65} the advantage to the host conferred by soluble fibrinogen in Fib\textsuperscript{AEK} mice over Fib\textsuperscript{−/−} mice in high-load \textit{S. aureus} peritonitis could be reversed to a disadvantage for the host following intravenous-induced \textit{S. aureus} bacteremia. Insights gained through more comprehensive studies of Fib\textsuperscript{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., D.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. 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.

From www.bloodjournal.org by guest on September 23, 2017. For personal use only.
References


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

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