A Phenome-Wide Association Study Uncovers a Pathological Role of Coagulation Factor X during *Acinetobacter baumannii* Infection

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ABSTRACT Coagulation and inflammation are interconnected, suggesting that coagulation plays a key role in the inflammatory response to pathogens. A phenome-wide association study (PheWAS) was used to identify clinical phenotypes of patients with a polymorphism in coagulation factor X. Patients with this single nucleotide polymorphism (SNP) were more likely to be hospitalized with hemostatic and infection-related disorders, suggesting that factor X contributes to the immune response to infection. To investigate this, we modeled infections by human pathogens in a mouse model of factor X deficiency. Factor X-deficient mice were protected from systemic *Acinetobacter baumannii* infection, suggesting that factor X plays a role in the immune response to *A. baumannii*. Factor X deficiency was associated with reduced cytokine and chemokine production and alterations in immune cell population during infection: factor X-deficient mice demonstrated increased abundance of neutrophils, macrophages, and effector T cells. Together, these results suggest that factor X activity is associated with an inefficient immune response and contributes to the pathology of *A. baumannii* infection.

KEYWORDS *Acinetobacter*, PheWAS, coagulation, innate immunity

Coagulation plays a considerable role in human physiology and thus, perturbations to coagulation affect many disease outcomes, including inflammation and immunity (1–4). Coagulation factor X (FX) is a vitamin K-dependent serine protease that has a prominent function in the coagulation cascade (1). FX is synthesized in the liver as an inactive zymogen and secreted into the bloodstream. Upon injury and coagulation pathway activation, a series of reactions converge on FX, which is cleaved into the active form, FXa. This protease interacts with other coagulation factors to convert prothrombin to its active form, thrombin (1). Thrombin converts fibrinogen into fibrin and leads to a stable blood clot (5).

While the biochemistry of FX has been well studied, the role of FX in coagulation-related physiology is an ever-expanding field. Clinical presentations of patients encoding FX variants are heterogeneous. Overt FX deficiency, which causes bleeding disorders that can range in severity from hemorrhagic symptoms to asymptomatic, can be hereditary or acquired as a result of disease (5). The homozygous and most severe hereditary forms of FX deficiency have an incidence rate of approximately one in a million (6) and are due to polymorphisms in the *F10* gene. Individuals with FX deficiency are continually being discovered, with different polymorphisms (7, 8). One

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well-known deficiency has been noted exclusively in the Friuli region of Italy: individuals carry a homozygous mutation in *F10* resulting in Pro343Ser. Affected individuals have a moderate bleeding tendency, with epistaxis, bleeding from the gums, posttraumatic hemarthroses, and bleeding after surgical procedures (9). Other congenital FX deficiencies have less overt clinical phenotypes (5) and underscore the opportunity to discover more about FX function and population variation in *F10*.

One method to identify genetic variation in patient populations, as well as associations between genotypes and clinical outcomes, is the use of phenome-wide association studies (PheWAS). PheWAS explore the association between nuclear single nucleotide polymorphisms (SNPs) and phenotypes represented in electronic health records (EHR) (10). PheWAS have successfully revealed novel SNP/phenotype associations and have been used to explore pleiotropy (11, 12), disease heritability (13), therapeutic and adverse medication effects (14, 15), and drug repurposing opportunities (16, 17).

There is extensive cross talk between coagulation and inflammation; therefore, coagulation plays a key role during bacterial infection (18). The formation of clots can entrap bacteria as a key physical defense, but a variety of bacteria have been found to induce coagulation cascades to subvert the clot (19). Some human innate immune cells also express protease activated receptors on their cell surfaces, which can be activated by coagulation proteases to initiate or amplify inflammation (20). Additionally, Gram-negative sepsis is classically associated with coagulopathies, especially acute disseminated intravascular coagulation (DIC) (21). *Acinetobacter baumannii* is a Gram-negative bacterium that has emerged as a frequent cause of hospital-acquired infections of the critically ill and as a leading cause of ventilator-associated pneumonia and burn wound infections (22). The majority of *A. baumannii* infections are caused by strains that are resistant to multiple classes of antibiotics, which leads to treatment failures (23). Thus, identifying novel or repurposing approved therapeutics that might aid the ability of the immune response to limit bacterial replication during infection is an attractive goal. In this study, we used PheWAS to identify clinical phenotypes associated with an SNP in FX (rs3211783), which provided insight into the association between FX and innate immune response to *A. baumannii* infection.

RESULTS

PheWAS identify predicted and novel disease associations with the *F10* SNP. In an attempt to detect phenotypes associated with alterations in FX function, the electronic health records (EHR) of approximately 30,000 patients and their associated exome sequencing were scanned for novel associations between ICD-9 medical codes and *F10* rs3211783 (see Table S1 in the supplemental material) (24). This SNP, not known to be in linkage disequilibrium with other variants, results in Gly192Arg in synthesized FX (position 149 in the mature secreted protein). Gly192 resides within the activation peptide of FX, close to the site at which factors IXa and VIIa cleave FX during clotting, releasing the activation peptide. This cleavage leads to FXa generation; therefore, Gly192 is not present in mature FXa (25). Experimental substitution of Gly192Arg did not affect coagulation and catalytic activity of the enzyme compared with those of the wild type (WT) (26); however, that *in vitro* study did not explore the direct effects of the SNP on the FX zymogen or any *in vivo* effects. While being catalytically active, the Gly192Arg variant may display *in vivo* defects such as reduced serum half-life; consequently, notable gaps in our knowledge regarding the overall functional effect of this SNP remain.

The Gly192Arg minor allele is found in about 3% of the world population and is most abundant in those of African descent (11%) (Table S1) (27). Despite the unknown impact of Gly192Arg, carriers of this SNP minor allele display an increased incidence of a number of hematological conditions in their EHR, compared to noncarriers, as indicated by odds ratios (ORs) of >1 (Table 1). These phenotypes were expected based on the known function of FX in blood clotting and thus support the integrity of the PheWAS results. Interestingly, PheWAS analysis also identified unexpected phenotypes involving the liver, which is the site of FX synthesis (Table 1).

TABLE 1 Phenotypes identified by PheWAS of *F10* rs3211783

Phenotypes	Condition	PheWAS code	P value	Odds ratio	No. of affected cases	Total no. of cases	No. of controls
Expected (validating) phenotypes	Other diseases of blood and blood-forming organs	289	1.2E−03	4.65	5	481	18,328
	Pernicious anemia	281.11	1.3E−02	6.41	2	195	15,680
	Hematemesis	578.1	2.5E−02	5.08	2	155	20,426
	Abnormality of red blood cells	289.9	3.3E−02	8.84	3	51	18,328
	Polycythemia vera, secondary	289.8	1.3E−04	10.25	3	188	20,349
Unexpected infection-related phenotypes	Mycoses	117	7.8E−07	8.62	6	278	21,953
	Athlete's foot	110.12	2.3E−05	13.19	3	106	21,953
	Cellulitis and abscess of trunk	681.7	2.7E−04	5.60	5	357	21,003
	Tuberculosis	010	3.2E−02	8.99	2	46	21,520
	Cholangitis	575.1	5.5E−03	5.24	3	221	23,497
	Endocarditis	420.3	2.7E−02	4.98	2	160	23,314
	Viral infection	079	3.9E−02	2.96	4	459	21,250
Unexpected liver-related phenotypes	Primary biliary cirrhosis	571.6	2.0E−07	9.91	6	232	19,711
	Other disorders of biliary tract	575.8	5.5E−04	6.05	4	251	23,497
	Other biliary tract disease	575	1.6E−03	3.88	6	587	23,497
	Jaundice (not of newborn)	573.5	2.0E−03	5.09	4	316	19,711
	Liver replaced by transplant	573.2	2.4E−03	4.92	4	327	19,711
	Other disorders of liver	573	2.7E−02	3.17	4	510	19,711
	Cirrhosis of liver without mention of alcohol	571.51	2.8E−02	2.84	5	709	19,711
	Chronic liver disease and cirrhosis	571	2.9E−02	2.42	7	1,149	19,711
	Malignant neoplasm of liver, primary	155.1	3.9E−02	4.47	2	174	21,458

In addition to the validating phenotypes, the PheWAS analysis revealed unexpected associations between Gly192Arg and a cluster of infection-related phenotypes, including endocarditis, mycoses, and cellulitis and abscess of the trunk (Table 1). Both phenotype clusters had positive ORs, indicating that SNP carriers are at an increased risk of developing these conditions. To better understand the pathogens driving a subset of the infectious phenotypes, we reviewed the available microbiology lab results of the infection-affected SNP carriers. Patients in this group had positive culture findings for a variety of pathogens (Table S2). Based on these observations, we hypothesized that FX may contribute to the innate immune response during bacterial infection, which might affect the host's ability to resist infection.

Factor X-deficient mice demonstrate enhanced bacterial burdens in the liver following systemic *Staphylococcus aureus* infection. To assess the role of FX during infection and leverage our PheWAS data toward potential novel therapeutics, we chose a model Gram-positive human pathogen, *Staphylococcus aureus*. To model the *F10* rs3211783 variant and FX deficiency in general, mice with the Friuli allele of *F10* (referred to as *F10*^{F/F} throughout), which have reduced FX activity, were used. As deletion of *F10* is an embryonic lethal mutation, this is the only known murine model of FX deficiency (28). We hypothesized that FX deficiency would cause an increase in bacterial replication over the course of infection, as humans with an FX mutation have increased rates of clinically coded bacterial infection. Age-matched wild-type C57BL/6J (referred to as B6 throughout) and *F10*^{F/F} mice were injected with *S. aureus* strain Newman via the retro-orbital venous sinus to establish systemic infection. At 96 h postinfection (hpi), mice were humanely euthanized and bacterial burdens were enumerated from infected organs. *F10*^{F/F} mice demonstrated a moderate increase in colonization of the liver (Fig. 1), suggesting that FX activity helps reduce *S. aureus* replication in the liver. These data are consistent with the results of the PheWAS and consistent with a model that FX activity helps protect from bacterial infection.

Factor X-deficient mice demonstrate reduced bacterial burdens after systemic *A. baumannii* infection. While the effect of FX deficiency on *S. aureus* infection was consistent with the PheWAS results, we also chose to model Gram-negative infection, as patients in the affected group had positive culture reports for both Gram-positive

FIG 1 Factor X-deficient mice demonstrate enhanced bacterial burdens in the liver following systemic *S. aureus* infection. Approximately 2×10^7 CFU of *S. aureus* Newman was injected via the retro-orbital venous sinus. Mice were euthanized and tissues removed at 96 hpi and bacterial burdens were quantified. Each dot represents a single mouse, and data are compiled from three experiments. Shown is the median with interquartile range; the y axis is set to the limit of detection. *, $P < 0.05$ by Mann-Whitney test.

and Gram-negative pathogens (Table S2). We chose *A. baumannii* as a model Gram-negative pathogen with limited clinical treatment options. Age- and sex-matched B6 and *F10^{F/F}* mice were injected with *A. baumannii* via the retro-orbital venous sinus to establish systemic infection. As early as 4 hpi, *F10^{F/F}* mice demonstrated reduced bacterial burdens in the blood (Fig. 2A), and at 24 hpi, fewer *A. baumannii* CFU were recovered from the blood and every organ tested for *F10^{F/F}* mice (Fig. 2B). These data suggest that the reduced FX activity in the *F10^{F/F}* mice results in less pathology during systemic *A. baumannii* infection. In this model, the magnitude of the difference in bacterial burdens between B6 and *F10^{F/F}* mice was much greater and across organs compared to that in infection with *S. aureus*; therefore, we sought to further understand the role of FX during *A. baumannii* infection.

An FDA-approved factor Xa inhibitor reduces bacterial burdens in B6 mice. To test whether pharmacological inhibition of FXa activity could reduce the bacterial burdens observed in B6 mice infected with *A. baumannii*, enoxaparin was administered at the time of infection. Enoxaparin binds antithrombin to irreversibly inactivate FXa (29, 30). Bacterial burdens were reduced in the heart, liver, and spleen in B6 mice after a single treatment with enoxaparin (Fig. 2C). These data are consistent with a pathological role for FX during systemic *A. baumannii* infection. However, pharmacological inhibition of FXa did not completely phenocopy the burdens of *F10^{F/F}* mice (Fig. 2B), either because of incomplete inhibition or because FXa-mediated clotting reactions are not completely responsible for differences observed between B6 and *F10^{F/F}* mice.

Factor X deficiency does not affect infection-induced coagulation. Gram-negative bacteria can induce clotting dysregulation, including microthrombi and DIC; therefore, we hypothesized that reduced FX activity in *F10^{F/F}* mice would protect from infection-induced hypercoagulation. Because naive *F10^{F/F}* mice have reduced coagulation activity (28), we reasoned that during infection, *F10^{F/F}* would not be subject to the pathology associated with hypercoagulation. However, standard measurements of coagulation parameters, such as thrombin generation time, do not capture any pathological effects of infection-induced coagulation and would be complicated by the difference in baseline coagulation parameter between genotypes. Therefore, we turned to tissue pathology to understand how mouse genotype impacts the effects of *A. baumannii*-induced coagulation. Histopathology of mock-infected and infected mice at 24 hpi detected no statistically significant differences between B6 and *F10^{F/F}* mice, measuring liver hepatitis and thrombosis and spleen lymphocytolysis and thrombosis

FIG 2 Factor X-deficient mice demonstrate reduced bacterial burdens after systemic *A. baumannii* infection. Approximately 2×10^8 to 3×10^8 CFU of *A. baumannii* 17978 was injected via the retro-orbital venous sinus. Mice were euthanized and tissues removed at 4 (A) and 24 (B) hpi and bacterial burdens were quantified. (C) Bacterial burdens 24 hpi following infection with approximately 2×10^8 CFU *A. baumannii* 17978 after treatment with PBS (vehicle) or 10 mg/kg of body weight of enoxaparin, intraperitoneally, at the time of infection. Each dot represents a single mouse, and data are compiled from two (A) or three (B and C) experiments. Shown is the median with interquartile range; the y axis is set to the limit of detection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Mann-Whitney test).

FIG 3 Factor X deficiency does not affect infection induced coagulation. Mice were injected with approximately 3×10^8 CFU of *A. baumannii* 17978 and euthanized at 24 hpi. Tissue were fixed and scored blindly (A) or hepatic thrombosis was quantified using anti-fibrin/fibrinogen immunohistochemistry (B). Each dot represents a single mouse, and data are compiled from a single experiment. For panel A, the whiskers of box-and-whisker plots indicate minimum to maximum. Horizontal lines in panel B indicate mean. No statistical difference ($P < 0.05$) was found by two-way analysis of variance (ANOVA) with Sidak's correction for multiple comparisons.

(Fig. 3A); however, a trend toward reduced liver hepatitis score and splenic lymphocytolysis and thrombosis was noted. To further assess *A. baumannii*-induced thrombosis, liver thrombus formation was measured using immunohistochemistry against fibrin, fibrinogen, and fibrinogen degradation products (fragments D and E). Again, no difference between mouse genotypes at 24 hpi was observed (Fig. 3B). These data suggest that, at least within the 24 hpi studied, *A. baumannii* infection did not exhibit an association with coagulation dysregulation in the B6 or *F10^{F/F}* mice.

***F10^{F/F}* mice demonstrate reduced serum cytokine and chemokine levels during *A. baumannii* infection.** As only modest differences were observed for infection-induced coagulation, we hypothesized that an altered immune response may account for the difference in bacterial burdens between B6 and *F10^{F/F}* mice. To test this hypothesis, cytokines and chemokines were quantified from serum at 4 and 24 hpi. *F10^{F/F}* mice exhibited reduced levels of a variety of cytokines by 24 hpi, including both proinflammatory (tumor necrosis factor [TNF] and interleukin 6 [IL-6]) and anti-inflammatory (IL-10) cytokines (Fig. 4A and Table S3). Chemokines, including the gamma interferon (IFN- γ)-induced CXCL10 and chemoattractants CXCL1, CXCL2, and CCL4 were reduced by 4 or 24 hpi (Fig. 4B and Table S3). This blunted cytokine and chemokine response in the infected *F10^{F/F}* mice coinciding with decreased bacterial burdens is consistent with a beneficial association of the altered immune response in the *F10^{F/F}* mice and *A. baumannii* infection.

FX activity is associated with a pathogenic immune response and mortality following injection of heat-killed *A. baumannii*. The reduction in cytokine and chemokine levels in *F10^{F/F}* mice could be the result of decreased bacterial burdens in the *F10^{F/F}* mice. To test this hypothesis, cytokines and chemokines were quantified at 24 h following injection with phosphate-buffered saline (PBS; mock infection) or heat-killed *A. baumannii*. For both mouse genotypes, the presence of heat-killed *A. baumannii* induced an immune response (Fig. 5A and B; Table S4). Similar to the case with live bacteria, *F10^{F/F}* mice injected with heat-killed *A. baumannii* experienced reduced serum cytokine and chemokine levels compared to those of B6 mice. Together, these data demonstrate that the blunted immune response in the *F10^{F/F}* mice is not the result of decreased bacterial burdens.

Therefore, we hypothesized that the inflammatory response in B6 mice is pathological during *A. baumannii* infection regardless of the ability of *A. baumannii* to replicate in organs. To examine this, heat-killed *A. baumannii* was injected into mice and survival was monitored for 8 days. B6 mice succumbed to this treatment at a greater rate than *F10^{F/F}* mice (Fig. 5C). Overall, we observed increased mortality during the systemic response to heat-killed *A. baumannii* in B6 mice compared to *F10^{F/F}* mice,

FIG 4 *F10^{F/F}* mice demonstrate reduced serum cytokine and chemokine levels during systemic *A. baumannii* infection. Serum cytokines (A) and chemoattractants (B) at 4 and 24 h following injection in the retro-orbital venous sinus of approximately 3×10^8 CFU of *A. baumannii* 17978 were quantified by Luminex analysis. Each dot represents data from a single mouse, from two experiments; the horizontal line indicates the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (one-way ANOVA with Sidak's correction for multiple comparisons, comparing genotypes at each time point). For graphing and statistical tests, values that exceeded or were below the limit of detection were set to the limit for each analyte.

suggesting that the heightened levels of cytokines and chemokines observed during *A. baumannii* infection are associated with normal FX activity and drive mortality. The survival of *F10^{F/F}* mice relative to the high mortality rate of B6 mice following injection of heat-killed *A. baumannii* suggested that *F10^{F/F}* mice might respond less robustly to a surface-associated molecular pattern of *A. baumannii*. Lipopolysaccharide (LPS) and the related molecule lipooligosaccharide (LOS) are obvious candidates. LPS alone is sufficient to induce coagulation in various models (19) and is a distinguishing feature of Gram-negative bacteria. Therefore, we sought to test whether the protection from *A. baumannii* infection in *F10^{F/F}* mice is a general phenomenon of Gram-negative infec-

FIG 5 FX activity is associated with a pathogenic immune response and mortality following injection of heat-killed *A. baumannii*. Serum cytokines (A) and chemoattractants (B) following retro-orbital injection with PBS (mock) or approximately 1×10^9 to 2×10^9 CFU of heat-killed *A. baumannii* ATCC 17978 were quantified by Luminex analysis after mice were euthanized at 24 hpi. (C) Survival of mice injected with approximately 1×10^9 to 2×10^9 CFU of heat-killed *A. baumannii* ATCC 17978. For panels A and B, each dot represents data from a single mouse, from two experiments; the horizontal line indicates the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (one-way ANOVA with Sidak's correction for multiple comparisons, comparing genotypes within each treatment group). For graphing and statistical tests, values that exceeded or were below the limit of detection were set to the limit for each analyte. For panel C, data are combined from two independent experiments ($n = 13$ for B6 mice and $n = 17$ for *F10^{F/F}* mice). *, $P < 0.05$ by log rank (Mantel-Cox) survival test.

tion. We infected B6 and *F10^{F/F}* mice with another opportunistic Gram-negative pathogen, *Pseudomonas aeruginosa*. *F10^{F/F}* mice were not protected from high bacterial burdens across organs following systemic infection with *P. aeruginosa* (Fig. S1). These data suggest that the pathology associated with FX activity during *A. baumannii* infection does not apply to all Gram-negative pathogens.

***F10^{F/F}* mice have increased numbers of proinflammatory immune cells during *A. baumannii* infection.** It is unclear how the broad reduction in cytokine and chemokine levels observed in the *F10^{F/F}* mice alters the recruitment of immune cells to

FIG 6 *F10^{F/F}* mice have increased numbers of proinflammatory immune cells during *A. baumannii* infection. Immune cells from spleen, kidneys, and blood of mice injected with either PBS (mock) or approximately 2×10^8 to 3×10^8 CFU of *A. baumannii* ATCC 17978 were quantified at 24 hpi using flow cytometry. (A) Neutrophils (CD11b⁺ Ly6G⁺ CD11c⁻ F4/80⁻) and dendritic cells (CD11b⁺ CD11c⁺ Ly6G⁻ F4/80⁻). (B) Macrophages (CD11b⁺ F4/80⁻ Ly6G⁻ CD11c⁻) and monocytes (CD11b⁺ F4/80⁻ Ly6G⁻ CD11c⁻). (C) CD8⁺ T_{eff} (CD4⁻ CD8⁺ C45RA⁺ CD62L⁻) and T_n (CD4⁻ CD8⁺ C45RA⁺ CD62L⁺) cells. (D) CD4⁺ T_{eff} (CD4⁺ CD8⁻ C45RA⁺ CD62L⁻), T_n (CD4⁺ CD8⁻ C45RA⁺ CD62L⁺), and T_{reg} (CD4⁺ CD8⁻ FOXP3⁺) cells. Each dot represents data from a single mouse, from two experiments. The horizontal line indicates the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (two-way ANOVA with Tukey's correction for multiple comparisons).

sites of infection. To understand the cellular response to *A. baumannii* in B6 and *F10^{F/F}* mice, we quantified myeloid and T cell populations at 24 hpi by flow cytometry (Fig. S2A), as they play critical roles in establishing the innate immune response. A variety of differences were observed between infected B6 and *F10^{F/F}* mice in the spleen, kidneys, and blood. The total number of myeloid cells (CD11b⁺) was slightly increased in the blood of *F10^{F/F}* mice and unchanged in the spleen and kidneys compared to those in B6 mice during infection (Fig. S2B). Further analysis of the myeloid cell population revealed a significant immune cell skewing, with increased neutrophils and decreased dendritic cells (DCs) in the spleen and blood of *F10^{F/F}* mice compared to B6 (Fig. 6 and Fig. S2C). Additionally, macrophage (M ϕ) numbers were increased in the blood of infected *F10^{F/F}* mice, coinciding with a decrease in monocyte numbers (Fig. 6B and Fig.

S2C). Monocyte numbers were increased in the spleens of *F10^{F/F}* mice; however, M ϕ numbers were unchanged compared to those in B6 mice. Surprisingly, myeloid cell populations were not altered in the kidneys of infected *F10^{F/F}* mice compared to those of B6 mice (Fig. 6A and B and Fig. S2C). Overall, increased numbers of neutrophils in the spleen and blood and increased numbers of M ϕ s in the blood of *F10^{F/F}* mice could enhance the containment of *A. baumannii* infection within these niches compared to that in B6 mice.

The total numbers of T cells were comparable between *F10^{F/F}* and B6 mice, with a small increase in the number of CD8⁺ T cells in the spleen and a small decrease in the total number of CD4⁺ T cells in the blood of *F10^{F/F}* mice (Fig. S3A). The T cell populations were skewed in the spleen and kidneys, with elevated numbers of CD8⁺ and CD4⁺ effector T cells (T_{eff} cells) in *F10^{F/F}* mice compared to those in B6 mice (Fig. 6C and D and Fig. S3B). CD8⁺ T_{eff} cells were decreased and CD4⁺ T_{eff} cells unchanged in the blood of *F10^{F/F}* mice compared to those in B6 mice. Coinciding with elevated CD8⁺ T_{eff} cells in the spleens and kidneys of *F10^{F/F}* mice, there were decreased numbers of naive T cells (T_n cells) (Fig. 6C and D and Fig. S3B). In the spleen, CD8⁺ T_n cells were also decreased in *F10^{F/F}* mice; however, the number of CD4⁺ T_n cells remained unchanged (Fig. 6C and D and Fig. S3B). Instead, the percentage of regulatory T cells (T_{reg} cells) was significantly reduced (Fig. 6C and D and Fig. S3B). Overall, the numbers of inflammatory myeloid and T cells were increased in *F10^{F/F}* mice; however, the alterations in the immune cell populations varied depending on the location within the mice. In the spleens of *F10^{F/F}* mice, the myeloid and T cell compartments were skewed, with increased neutrophil and CD4⁺ and CD8⁺ T_{eff} cell numbers. In contrast, only the myeloid compartment seemed to be altered in the blood of *F10^{F/F}* mice, with increased neutrophils and M ϕ s, while only the T cell compartment was altered in the kidneys, with increased numbers of CD4⁺ and CD8⁺ T_{eff} cells and decreased numbers of T_{reg} cells. Taken together, the results show that during infection, *F10^{F/F}* mice exhibit increased numbers of neutrophils, M ϕ s, and T_{eff} cells, which likely contribute to the reduced bacterial burdens at 24 hpi previously observed.

DISCUSSION

In this study, we discovered associations between a polymorphism in a known drug target and novel disease phenotypes using PheWAS. Individuals with a SNP in *F10* demonstrated validating phenotypes of hemostatic disorders but also increased odds ratios of being hospitalized for a variety of infection-related disorders (Table 1). This finding was consistent with the mouse model of FX deficiency, in which FX-deficient mice demonstrated greater bacterial burdens in the liver after infection with *S. aureus* (Fig. 1). However, infection with *A. baumannii* showed an opposite effect: FX-deficient mice displayed enhanced clearance of *A. baumannii* (Fig. 2). Together, these findings suggest that FX plays an important role in antibacterial immunity, but in a pathogen-specific manner. It is possible that the results of the *S. aureus* infection are complicated by the multiple *S. aureus* virulence factors that can modulate coagulation, including coagulase and von Willebrand factor-binding protein (31). However, combined with the results obtained from the *P. aeruginosa* infection (Fig. S1), the finding that FX is pathological during *A. baumannii* infection may be unique to *A. baumannii* or specific pathogens based on the type of immune activation and/or ability to affect coagulation elaborated during infection by those pathogens.

It is important to note that the *F10^{F/F}* mouse model of FX deficiency has its shortcomings as a model for the human SNP uncovered by PheWAS. The exact clotting parameters and any defect in coagulation associated with Gly192Arg are unknown. However, patients with this SNP had associations with modest hematological conditions (Table 1), suggestive of Gly192Arg affecting some properties of FX *in vivo*. Unfortunately, there is lack of experimental data on the hemostatic effects of the Gly192Arg *in vivo*. However, data from other human mutations leading to FX deficiency point to variable bleeding tendencies, and it is extremely rare to observe a human with <1% FX activity. Therefore, we believe that the *F10^{F/F}* animals used in this study are not

only the only genetic model for FX deficiency, but importantly, they model the absence of severe bleeding phenotypes seen in humans with FX deficiency. This may include those patients with the Gly192Arg mutation.

As a proof of concept for the use of PheWAS to drive novel therapeutic development, we found that the FDA-approved FXa inhibitor enoxaparin was able to partly reduce bacterial burdens in B6 mice, suggesting that anticoagulants might offer new adjunctive treatment in specific clinical situations, such as for *A. baumannii* sepsis (Fig. 2C) or bacterial cholangitis, endocarditis, or mycoses (Table 1). While treatment with enoxaparin was shown to be an effective inhibitor of coagulation in mice (32), further work identifying the best inhibitor of murine FX, as well as optimal treatment times and delivery route, should be performed to understand how modulation of FX with FDA-approved small molecules could be applied to bacterial infections.

Heat-killed *A. baumannii* induces a strong cytokine and chemokine response, which is blunted in FX-deficient mice, coinciding with increased survival relative to that of B6 mice (Fig. 5). Together, these data suggest that FX activity associates with an immune response that is pathological during systemic *A. baumannii* infection. We had hypothesized that LPS/LOS of *A. baumannii* induced a cytokine response and pathological clotting in B6 mice, which would still occur with heat-killed bacteria. There was no protection of *F10^{F/F}* mice following infection with *P. aeruginosa*, suggesting that the pathology of FX activity is not true for all Gram-negative infections. However, it is still possible that the lipooligosaccharide of *A. baumannii* is responsible for this unique phenotype observed in this study. As *A. baumannii* is known to incorporate LOS in its outer membrane, and it is capable of modifying its outer membrane and even surviving without LOS (33), the exact mechanisms by which *A. baumannii* activates coagulation and immune signaling remains to be investigated. While the mechanism is not clear, our work indicates that the interplay between FX and clot formation is not completely responsible for this response, as only minor changes were detected between mouse genotypes when assessing pathogen-induced hypercoagulation (Fig. 3). Instead, we detected widespread changes to the immune cell populations (Fig. 6) that suggest that *F10^{F/F}* mice have a more targeted immune response, which limits bacterial replication.

It is challenging to understand how a reduction in the number of cytokines and chemokines results in a stronger, more targeted immune response in *F10^{F/F}* mice. The enhanced numbers of inflammatory immune cells across the organs in the presence of decreased cytokines and chemokines could indicate that the activation of the innate immune response is more efficient in *F10^{F/F}* mice and results in rapid clearance of *A. baumannii* during infection. However, reduced chemokines and cytokines in *F10^{F/F}* mice are observed even when the mice are injected with heat-killed bacteria, suggesting that viable bacteria are not required for this phenomenon. Coagulation and inflammation are invariably linked; however, the role of FXa activity is not completely clear (18). Indeed, studies on FXa as an endothelial barrier protective or disruptive mediator are discrepant (34–38). While our data do not directly address these FXa functions, our observations are consistent with a link between reduced FX activity (in the *F10^{F/F}* mice) and enhanced efficiency of immune cell activation. Our findings suggest that FX activity may dull the inflammatory response in myeloid and T cells during infection with *A. baumannii*. In *F10^{F/F}* mice, the inhibitory effect of FX is removed, and there is a greater enhancement in neutrophil, M ϕ , and T_{eff} cell numbers. Interestingly, each organ seems to provide a unique immunological niche, so each organ in the *F10^{F/F}* mice exhibits a different skewing of immune cells, which suggests that FX plays pleiotropic roles during infection dependent on the organ. Further studies are necessary to unravel the complexities of FX and determine how it influences the immune response during infection.

The interplay between FX and antibacterial immunity adds to a growing body of literature indicating that FX, and coagulation more broadly, is a critical determinant of the immune response to bacterial or viral infection. For instance, factor XII-deficient mice are protected in *Klebsiella pneumoniae* pneumonia, but factor XI-deficient mice are more susceptible to *K. pneumoniae* pneumonia (50). Additionally, factor XI-deficient

mice have increased survival in models of cecal ligation and puncture sepsis (39, 40). Interestingly, the survival advantage was partially attributed to changes in the cytokine response, where *FXI*^{-/-} mice demonstrated reduced TNF and IL-10 induction compared to WT mice (39), as observed in our study. Consistent with our study, inhibition of FXa and thrombin was shown to improve outcomes of Gram-negative sepsis in baboons by attenuating DIC and inhibiting IL-6 induction (38). Taken together, the findings show that coagulation appears to contribute to defense against bacterial infection in a variety of ways. Further work is required to understand the mechanisms by which FX affects cytokine release and innate immune cell populations during bacterial infection, and how this might be exploited therapeutically. Together, our results highlight the potential use of human clinical PheWAS to generate hypotheses, drive basic discovery using laboratory models, and validate possible novel treatments.

MATERIALS AND METHODS

PheWAS. The BioVU biorepository at Vanderbilt University Medical Center contains approximately 250,000 deidentified DNA samples extracted from excess patient blood samples collected during routine clinical testing that would otherwise be discarded. The specimens are linked to corresponding, longitudinal clinical and demographic data derived from the Synthetic Derivative, a deidentified EHR built for research purposes (41–43). PheWAS were conducted using previously reported methods (10, 15, 44, 45); analysis was focused on 29,722 patients of European ancestry in BioVU genotyped using the Illumina Infinium Exomechip (Illumina, Inc., San Diego, CA) and phenotype data extracted from deidentified electronic health records to achieve maximum statistical power. For the PheWAS analysis, all distinct ICD-9 billing codes were captured from each patient's record and translated into corresponding phenotype groupings. A case was defined as a record that has two or more ICD-9 codes that maps to one of the approximately 1,660 PheWAS phenotypes. Patients with records that did not contain any ICD-9 codes belonging to the exclusion code grouping corresponding for that case were designated controls. The PheWAS algorithm was then applied to calculate case and control genotype distributions, the χ^2 distribution, and associated allelic *P* value and allelic odds ratio (OR). For those χ^2 distributions in which observed cell counts fell below five, Fisher's exact test was used to calculate the *P* value using the R statistical package (<http://www.r-project.org/>). Only phenotypes that occurred in a minimum of 25 cases (0.42% of genotyped patients) were included in the analysis (10). The *P* values displayed in Table 1 are not subject to acorrection for multiple comparisons.

The variant of focus for these studies was the *F10* missense SNP rs3211783 (Gly192Arg). This variant was selected based on the presence of meaningful validation signals in the PheWAS results to support inference of variant effects *in vivo*, including bleeding and coagulopathy phenotypes expected to be observed if altered factor X function was present, as described further in the Results. Given the gaps in the literature regarding potential effects of this variant, we pursued work in an animal model to explore the hypotheses generated from our PheWAS findings.

Mice. FX Friuli [*F10*^{F/F}] mouse (28) sperm was provided by Paris Margaritis and rederived in the C57BL/6J (Jackson) background by the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Shared Resource. Mice were genotyped according to the method of Tai (28) and maintained as a homozygous colony. *F10*^{F/F} mice have normal FX antigen levels but FX activity levels similar to those of homozygous affected humans (~5%); this level is sufficient to rescue embryonic and postnatal lethality (28). C57BL/6J mice (Jackson) were used as wild-type comparators.

Mice were housed in specific-pathogen-free housing, with water and chow provided *ad libitum*, in a 12-h:12-h light:dark cycle. Mice were in cages, separated by sex, in groups of 3 to 5 (typically 5). For *A. baumannii* infections, mice were approximately 7 weeks old at the time of infection, and females were used throughout, except for Fig. 3B and Fig. 6, which show data from males and females of both genotypes. For *S. aureus* infection, mice were approximately 8 weeks old and a mixture of males and females were used. Female mice weighed between 16 and 22 g and male mice weighed between 19 and 26 g at the time of infection; infections were carried out between 10 a.m. and 1 p.m. For anesthesia, 2,2,2-tribromoethanol (Avertin) was dissolved in *tert*-amyl alcohol at 1 mg/ml, diluted in sterile PBS to 1.25%, and sterilized with a 0.44- μ m syringe filter; approximately 220 μ l was administered by intraperitoneal injection. Mice were not deidentified on genotype or treatment group during experiments, but samples were deidentified for pathology and flow cytometry experiments. The number of mice per experiment group and the number of independent experiments are listed in each figure legend. Statistical analysis of mouse infections are listed in figure legends. All infections were performed at the Vanderbilt University Medical Center under the principles and guidelines described in the *Guide for the Care and Use of Laboratory Animals* (46) using Institutional Animal Care and Use Committee (IACUC)-approved protocol M1600123-00. Vanderbilt University Medical Center is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility and registered with the Office of Laboratory Animal Welfare (OLAW), assurance number A-3227-01.

***S. aureus* infection.** *S. aureus* Newman (47) was streaked to tryptic soy agar (TSA) from a -80°C stock. After 24 h at 37°C, a single colony was used to inoculate 3 ml of tryptic soy broth (TSB) in a 15-ml aeration culture tube, and grown overnight for 16 h at 37°C with shaking. Fifty microliters of this culture was inoculated into 5 ml of TSB in a 15-ml conical tube and grown for 3.5 h at 37°C on a rotary drum. Bacteria were collected by centrifugation, washed twice in ice-cold PBS, and resuspended in ice-cold PBS.

to a concentration of 2×10^8 CFU/ml. For each infection, the inoculum was serially diluted in PBS and plated to TSA for enumeration.

Mice were anesthetized and subsequently injected with 100 μ l of the *S. aureus* suspension into the retro-orbital venous sinus. For bacterial burden enumeration, mice were humanely euthanized at the desired times by CO₂ inhalation, and organs were sterilely collected in PBS, homogenized using a Next Advance Bullet Blender (navy lysis tubes), serially diluted in PBS, and plated to TSA for enumeration.

A. baumannii infection. *A. baumannii* ATCC 17978 was streaked to lysogeny broth agar (LBA) from a -80°C stock. After 24 h at 37°C , a single colony was used to inoculate 3 ml of LB in a 15-ml aeration culture tube and grown overnight for 16 h at 37°C with shaking. Ten microliters of this culture was inoculated into 10 ml of LB in a 50-ml conical tube and grown for 3.5 h at 37°C with shaking. Bacteria were collected by centrifugation, washed twice in ice-cold PBS, and resuspended in ice-cold PBS to a concentration of 1×10^9 to 3×10^9 CFU/ml, as described in the figure legends. For each infection, the inoculum was serially diluted in PBS and plated to LBA for enumeration.

Mice were anesthetized and subsequently injected with 100 μ l of the *A. baumannii* suspension into the retro-orbital venous sinus. For bacterial burden enumeration, mice were humanely euthanized at the desired times by CO₂ inhalation, and organs were sterilely collected in PBS, homogenized using a Next Advance Bullet Blender (navy lysis tubes), serially diluted in PBS, and plated to LBA for enumeration. For survival experiments, mice were infected as described above, weighed daily, and monitored daily for death or moribund status. For enoxaparin treatment, B6 (Jackson) mice were anesthetized and then 100 μ l of either PBS or 1.9 mg/ml of enoxaparin (Fresenius Kabi; number FK562586) for a final dose of approximately 10 mg/kg (32) was injected into the intraperitoneal cavity. One hundred microliters of *A. baumannii* ATCC 17978 in PBS for a final inoculation of 2×10^8 to 3×10^8 CFU was injected into the retro-orbital venous sinus.

For infection with heat-killed bacteria, *A. baumannii* was grown and prepared as described above, except that the organism was resuspended in PBS to a final density of approximately 2×10^{10} CFU/ml, heated in a 70°C water bath for 45 min, cooled to room temperature, and then placed on ice. A portion of the inoculum was plated to LBA to ensure that no viable bacteria were detected.

Pathology and immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin immediately following euthanasia of the mice. Unbiased, standardized sectioning of the liver (48) was performed and spleens were submitted whole for histological processing. Fixed tissues were routinely processed using a standard 8-h processing cycle of graded alcohols, xylenes, and paraffin wax, embedded and sectioned at 4 to 5 μm , floated on a water bath, and mounted on positively charged or hydrophobic glass slides.

Hematoxylin and eosin (H&E) staining was performed on a Gemini autostainer (Thermo Fisher Scientific, Waltham, MA). Immunohistochemical (IHC) staining for thrombosis was performed using an anti-fibrinogen/fibrin (FGA) antibody on a Leica Bond-Max IHC autostainer (Leica Biosystems Inc., Buffalo Grove, IL). All steps besides dehydration, clearing, and coverslipping were performed on the Bond-Max. Slides were deparaffinized. Enzyme-induced antigen retrieval was performed using proteinase K (Dako/Agilent, Carpinteria, CA) for 5 min. Slides were incubated with anti-FGA (A0080; Dako/Agilent Technologies, Inc.) for 1 h at a 1:5,000 dilution. The Bond Polymer Refine Detection system was used for visualization. Slides were then dehydrated, cleared, and coverslipped.

All histopathologic interpretation was conducted by a board-certified veterinary pathologist under masked conditions. Semiquantitative lesion scoring in H&E-stained sections of the liver (hepatitis [49] and thrombosis) and spleen (lymphocytolysis and thrombosis) was completed on a 5-point scale. Quantitation of hepatic thrombosis was performed on digitized, FGA-stained IHC slides using the Aperio ImageScope positive pixel count v9 algorithm.

Cytokine and chemokine analysis. Cytokines were quantified using a Milliplex MAP mouse cytokine/chemokine magnetic kit (Millipore; number MCYTOMAG-70K-PMK; premixed beads, number MCYPMK25-MAG) multiplex assays according to the manufacturer's instructions and analyzed with a Luminex FLEXMAP 3D instrument. Tissues were removed immediately following euthanasia and placed in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and homogenized using a Next Advance Bullet Blender (navy lysis tubes), and protein content was quantified by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA). Whole blood was collected by blind cardiac puncture following euthanasia and allowed to clot at room temperature for at least 30 min, and then serum was collected by centrifugation at $6,500 \times g$ for 10 min at 4°C . All samples were stored at -80°C until analysis. Tissue samples were analyzed undiluted and cytokine values were normalized to total protein content, while serum was diluted 1:1 in serum matrix buffer, except for samples of limited volume, which were diluted 1:2. Analyte values were corrected for dilution.

Flow cytometry. Organs and blood were harvested from mice and single cell suspensions were generated from the kidneys and spleen. Red blood cells were lysed from organ cell suspensions and peripheral blood, followed by the introduction of a LIVE/DEAD stain (Invitrogen; L23105) on ice for 20 min. Samples were centrifuged 4 min at $\sim 800 \times g$, aspirated, fixed in 4% paraformaldehyde at room temperature, and transferred onto ice for 15 min. Samples were centrifuged for 4 min at $\sim 800 \times g$, aspirated, and resuspended in mouse Fc Block (BD Pharmingen; clone 2.4G2) diluted in fluorescence-activated cell sorting (FACS) medium (PBS plus 2% FBS and 0.02% sodium azide) for 20 min on ice. Following blocking, samples were centrifuged for 4 min at $\sim 800 \times g$, aspirated, and resuspended in FACS medium containing anti-CD11b, -CD11c, -Ly6G, -F4/80, -CD4, -CD8, -CD45RA, and -CD62L (Biolegend) for 20 min on ice. To stain for T_{reg} cells, samples were centrifuged for 4 min at $\sim 800 \times g$, aspirated, and resuspended in saponin permeabilization buffer (PBS plus 0.05% saponin and 0.5% bovine serum albumin [BSA]) containing anti-FOXP3 (Biolegend). Finally, samples were centrifuged for 4 min at

~800 × g, aspirated, and resuspended in FACS medium for flow cytometry. Data were collected using a BD LSRII flow cytometer with FACSDIVA software and analyzed using FlowJo (FlowJo LLC, Ashland, OR).

***Pseudomonas aeruginosa* infection.** *P. aeruginosa* PAO1 was streaked to lysogeny broth agar (LBA) from a −80°C stock. After 24 h at 37°C, a single colony was used to inoculate 3 ml of LB in a 15-ml aeration culture tube and grown overnight for 16 h at 37°C with shaking. One hundred microliters of this culture was inoculated into 10 ml of LB in a 50-ml conical tube and grown for 3.5 h at 37°C with shaking. Bacteria were collected by centrifugation, washed twice in ice-cold PBS, and resuspended in ice-cold PBS to a concentration of 6 × 10⁸ CFU/ml. For the infection, the inoculum was serially diluted in PBS and plated to LBA for enumeration. Female mice, approximately 7 weeks old, were infected and harvested as described for *A. baumannii* infections above.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00031-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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