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ORIGINAL ARTICLE

An antibody targeting high-molecular-weight kininogen blocks contact system activation in a model of polymicrobial sepsis

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Abstract

Background: Polymicrobial sepsis is an infectious disease characterized by excessive inflammation and coagulation that is linked to more severe disease pathology, organ failure, and fatality. The plasma contact system is a protein cascade in the blood that can be activated by bacteria and contributes to both inflammation and coagulation.

Objectives: To determine if inhibiting the plasma contact system by targeting high-molecular-weight kininogen (HK) can exert a protective effect on bacteria-induced coagulation.

Methods: Polymicrobial cecal slurry (CS) was prepared from donor mice and used for *ex vivo* and *in vivo* experiments. CS was used *in vivo* to establish a murine model of polymicrobial sepsis. CS was incubated with mouse or human plasma *ex vivo*. Contact system activation was assessed by Western blot, and clotting was assessed spectroscopically. Our monoclonal antihuman HK antibody, 3E8, was used to determine how contact system inhibition could delay CS-induced coagulation *ex vivo*.

Results: Polymicrobial CS activated the plasma contact system *in vivo* in mice and *ex vivo* in both mouse and human plasma. CS promoted coagulation in mouse and human plasma *ex vivo*. Treatment with our 3E8 anti-HK antibody protected against CS-induced contact system activation and coagulation.

Conclusion: The plasma contact system was activated in the CS model of polymicrobial sepsis. Targeting HK in polymicrobial sepsis may have beneficial effects in limiting excessive coagulation and could represent a novel therapeutic avenue to promote survival in sepsis.

KEYWORDS
blood coagulation, high-molecular-weight kininogen, kallikrein-kinin system, sepsis

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1 | INTRODUCTION

The plasma contact system is initiated by the activation of coagulation factor (F)XII by negatively charged surfaces. When activated, this system initiates the coagulation cascade via FXI, resulting in vessel occlusion, hypoperfusion, and microinfarcts. The contact system can also promote activation of the proinflammatory pathway via plasma prekallikrein (PK), resulting in the cleavage of high-molecular-weight kininogen (HK) and the release of bradykinin (BK), which triggers increased vascular permeability and inflammation. The contact system has been implicated in numerous diseases, including those with infectious, vascular, and/or inflammatory pathologies [1–3]. Of note, both gram-negative and gram-positive bacteria have been shown to activate the contact system [4–12]. Severe cases of infection, in which bacteria elicit a pathological host response, can result in an often fatal condition known as sepsis. Sepsis-induced fatality is thought to be driven, at least in part, by dysregulated excessive coagulation, which can lead to disseminated intravascular coagulation, inflammation, and organ failure (reviewed in Raghunathan et al. [13]). The extent to which bacterial activation of the contact system contributes to the pathological hypercoagulation, BK-mediated hypotension, and inflammation observed in sepsis remains unknown.

Since the contact system initiates 2 separate proteolytic cascades, preventing the initial step (activation of FXII to activated FXII [FXIIa]) would seem to be the most promising target for intervention. However, in multiple studies, depletion of HK protects mice, whereas depletion of FXII does not [14–18]. This may be explained by the observation that FXI and PK circulate in complex with HK, which accelerates their activation by FXIIa [19,20]. Since activated PK (PKa) plays a critical role in feedback activation of FXII, preventing PK activation also blocks continued FXII activation. Furthermore, as many proteases can either activate the contact system downstream of FXII or directly cleave HK to release BK [21,22], inhibiting FXII activity would not completely shut down the system.

We have previously shown that HK is a suitable target to prevent activation of both FXI and PK, thereby limiting both inflammation and coagulation [23–25]. Studies using our previously developed monoclonal antihuman HK antibody, 3E8, have found that treatment with 3E8: (1) blocks FXI and PK binding to HK and, therefore, prevents their activation, (2) inhibits HK cleavage and BK generation, (3) prevents feedback activation of FXII, and thus (4) reduces clotting and inflammatory activity [23–25]. Therefore, 3E8 anti-HK antibody represents a possible therapy for pathologies that involve contact system activation. Notably, neither HK, PK, nor FXII deficiency is associated with bleeding [26].

In this study, we show that the CS model of polymicrobial sepsis activates the plasma contact system *in vivo* and promotes clotting *ex vivo*. Importantly, we demonstrate that treatment with our monoclonal 3E8 anti-HK antibody protects against CS-induced HK cleavage, contact system activation, and clotting in human plasma *ex vivo*. Our results reveal a novel therapeutic strategy for reducing inflammation, coagulation, and fatality in sepsis.

2 | METHODS

2.1 | Mice

All animal experiments were conducted in accordance with the guidelines of the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval from the Animal Care and Use Committee of The Rockefeller University. C57Bl/6J wild-type (WT) mice were obtained from The Jackson Laboratory (strain #000664) and maintained and bred in Rockefeller's Comparative Bioscience Center. Adult (10-14 weeks old) female littermates were used for murine *in vivo* CS experiments. Cecal contents were collected from adult male WT littermates.

2.2 | CS model of polymicrobial sepsis

CS was prepared as previously described [27,28] from the pooled cecal contents of 10 WT male mice. The final CS stock was frozen in 15% glycerol in phosphate-buffered saline (PBS) at a concentration of 100 mg/mL. CS was then aliquoted into cryovials and frozen in a cryogenic freezing container and stored at −80 °C until needed. For experiments involving heat-inactivated CS (HI-CS), CS was heated at 65 °C for 20 minutes. CS aliquots were rapidly thawed in a warm water bath when needed. For *in vivo* experiments, CS was thawed and diluted (1:2) in PBS. Diluted CS was administered at a dose of 13 μ L/g of body weight via intraperitoneal (i.p.) injection. Control mice received vehicle (7.5% glycerol in PBS) i.p. injection at a dose of 13 μ L/g of body weight. Mice were checked twice daily and monitored for changes in weight and disease activity index (weight loss, posture, fur, and activity; adapted from Cooke et al. [29]) to determine disease severity.

2.3 | Blood and plasma collection and analysis

Mice were anesthetized, and whole blood was collected by cardiac puncture into 110 mM sodium citrate at a ratio of 9:1. Platelet-poor plasma was prepared by centrifuging twice at 1750 × *g* for 15 minutes. For clotting and *ex vivo* experiments, plasma samples from 5 to 10 mice were pooled when appropriate. Pooled citrated human plasma and plasma with congenital deficiencies of clotting factors were purchased from George King Bio-Medical, Inc. In a separate cohort of mice, whole blood was collected into EDTA-coated tubes, and complete blood count panels were performed by the Laboratory of Comparative Pathology at Memorial Sloane Kettering Cancer Center.

For *ex vivo* analysis of contact system activation, pooled citrated mouse or human plasma was incubated with HEPES buffer (20 mM HEPES, 140 mM NaCl), vehicle, or CS as labeled for 1 hour at 37 °C with rotation. To test the ability of 3E8 anti-HK antibody to inhibit CS-induced contact system activation, plasma was preincubated with 3E8 (3 μ M) for 30 minutes at 37 °C with rotation. Contact system activation was determined by Western blotting.

2.4 | Western blotting

Plasma was diluted in Laemmli Buffer (Bio-Rad) and heated to 95 °C for 5 minutes. Equal volumes of plasma were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and analyzed using antibodies against FXII/FXIIa (CL20055AP, Cedarlane Labs), human PK (SAPK-AP, Affinity Biological), mouse PK/PKa (AF2498, R&D System), human HK (Ab124737, Abcam), mouse HK (MAB22061, R&D Systems), and transferrin (AB82411, Abcam). Blots were imaged via Bio-Rad ChemiDoc. Protein levels were quantified by densitometry with ImageJ (National Institutes of Health). All experiments were repeated at least 3 times.

For *ex vivo* analysis of CS-induced activation of the plasma contact system, plasma was incubated with CS or HI-CS at the designated dilutions for 1 hour at 37 °C. For analysis of 3E8-mediated inhibition of contact system activation, plasma was preincubated with 3E8 (3 μ M) for 30 minutes at 37 °C with rotation prior to the addition of CS at designated dilutions (Supplementary Table).

2.5 | Clotting assays

Pooled citrated mouse plasma (BioChemed) or human plasma (normal pooled plasma [NPP] or plasma with congenital coagulation factor deficiencies; George King Bio-Medical, Inc) was used for *ex vivo* clotting assays and run as previously described [30,31]. Plasma was diluted in HEPES buffer in a 96-well microtiter plate. Activation of the intrinsic pathway was initiated by adding activated partial thromboplastin time (APTT) reagent solution (APTT-XL; Pacific Hemostasis), an ellagic acid-based activator, and activation of the extrinsic pathway was initiated using a prothrombin time (PT) reagent (Thromboplastin-D [Pacific Hemostasis] with 25 mM $CaCl_2$). Clot formation was induced by the addition of CS. To measure clot formation, absorbance readings were recorded every 5 seconds at 350 nm in a microtiter plate reader (SpectraMax Plus 384; Molecular Devices). Clotting time was determined as time to reach ½ maximum. In select experiments, corn trypsin inhibitor (CTI) was used at a concentration of 2.66 μ M, and 3E8 was used at a concentration of 670 nM [30].

2.6 | Chromogenic activity assay

FXIIa/PKa (S2302, DiaPharma) and FXI (S2366, DiaPharma) activities were measured by chromogenic assays. Pooled citrated mouse plasma, human NPP, or coagulation factor-deficient human plasma was incubated with CS or HI-CS and HEPES buffer for 5 minutes at 37 °C. Chromogenic substrate (0.67 mM) was added to the samples, and factor activity was measured in kinetic mode at 405 nm using a spectrophotometer (Molecular Devices). The experiments were performed in triplicate using 96-well plates. Activity bar graphs were prepared from cumulative data from different experiments at indicated time points.

2.7 | Statistical analysis

All statistical analyses were performed using GraphPad Prism software v10.

3 | RESULTS

3.1 | Polymicrobial CS activates the plasma contact system and induces clotting in mouse plasma *ex vivo*

The plasma contact system can be activated by negatively charged substances, including bacteria. Thus, we sought to determine if polymicrobial CS could activate the plasma contact system *ex vivo*. We found that the addition of CS to mouse plasma led to the cleavage of HK and the activation of PK to PKa (Figure 1A). Activation of the plasma contact system can promote coagulation via the intrinsic clotting pathway. Using an *ex vivo* clotting assay, we determined that CS accelerated *ex vivo* clotting in pooled citrated mouse plasma in a dose-dependent manner (Figure 1B). Of note, vehicle had no effect on plasma contact system activation or clotting (Figure 1A, B). These results show that CS can directly activate the mouse plasma contact system and induce clotting, even in the absence of platelets and the host immunological response.

3.2 | CS model of polymicrobial sepsis activates the plasma contact system in mice *in vivo*

In vivo polymicrobial sepsis was induced by i.p. injection of CS into WT mice. Twenty-four hours after injection, mice that received CS showed significant weight loss and increased sickness behavior as determined by the disease activity index [29], which scores changes in posture, activity, weight, and appearance (Figure 2A). Mice that received CS had reduced circulating platelet counts (Figure 2B) and delayed intrinsic (ie, APTT) and extrinsic (ie, PT) pathway-associated clotting (Figure 2C), reflective of consumption of coagulation factors and increased coagulation in response to the septic stimulus *in vivo* [32]. Septic mice showed increased activation of the plasma contact system as determined by a reduction in plasma levels of intact HK, PK, and FXII compared with mice that received vehicle (Figure 2D). These results indicate that CS can activate the plasma contact system and coagulation factors to induce clotting *in vivo*.

3.3 | Polymicrobial CS activates the plasma contact system and induces clotting in human plasma *ex vivo*

As observed with mouse plasma, incubation of human citrated NPP with CS showed a dose-dependent activation of the plasma contact system. CS induced the cleavage of HK, the activation of PK, and the

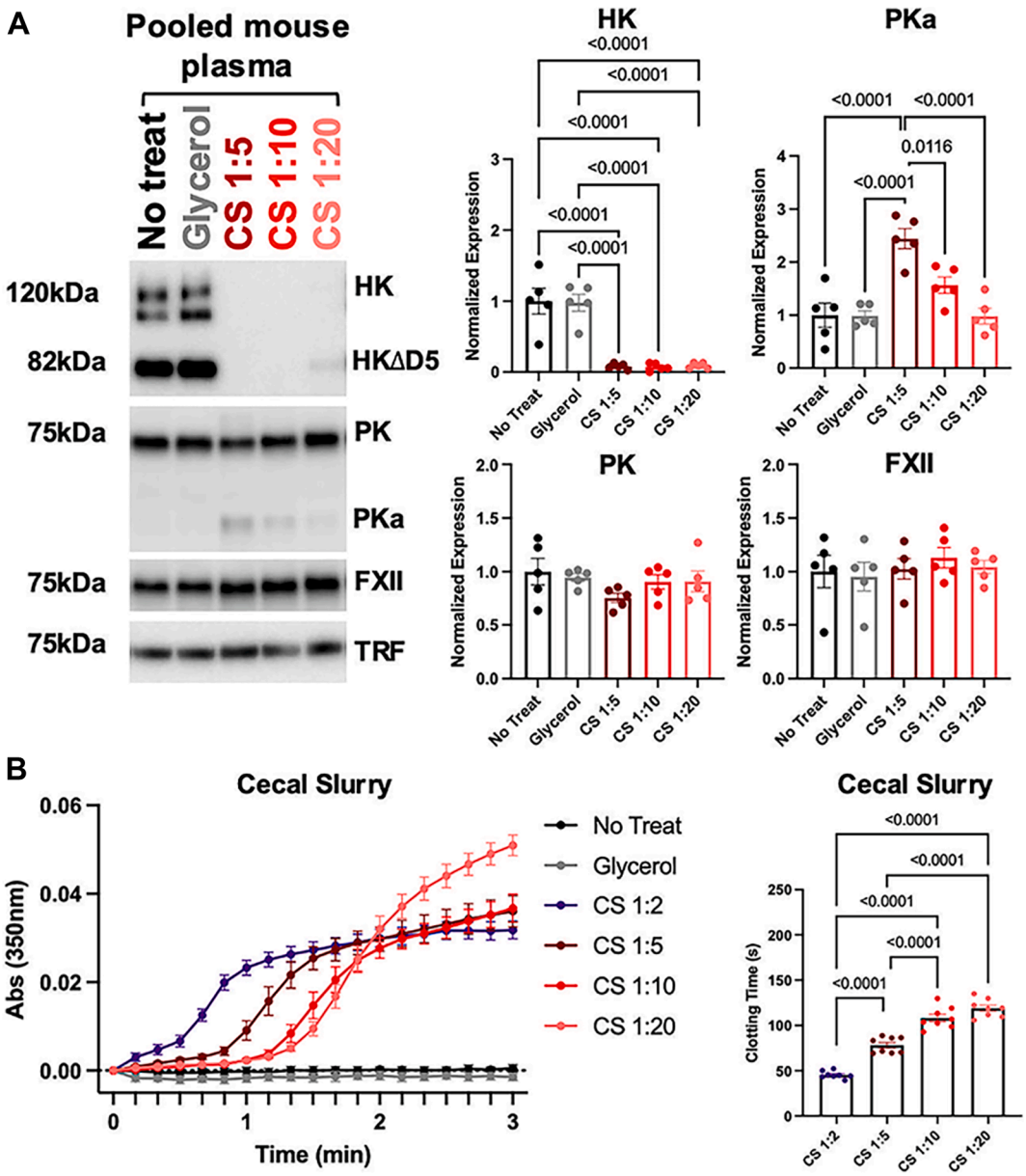

FIGURE 1 Cecal slurry (CS) activates the plasma contact system and induces clotting in mouse plasma *ex vivo*. Pooled citrated plasma from wild-type mice was incubated with buffer (No Treat), vehicle (glycerol), or CS at the indicated dilution for 1 hour at 37 °C. (A) Left, representative Western blot. Right, quantification of levels of high-molecular-weight kininogen (HK) and HK isoform lacking domain 5 (HKΔD5), prekallikrein (PK), activated PK (PKa), and factor (F)XII normalized to transferrin (TRF) after incubation with CS. *n* = 5 per group; one-way analysis of variance (ANOVA) with Tukey's post hoc test. (B) CS-induced clotting in pooled mouse plasma in a dose-dependent manner. No Treat represents negative control, and glycerol is the vehicle control. Left, plot shows clotting as determined by absorbance (Abs) at 350 nm over time. Right, bar graph shows clotting time of pooled mouse plasma when induced by varying dilutions of CS. *n* = 8 per group, one-way ANOVA with Tukey's post hoc test. Data are shown as mean ± SEM.

generation of PKa/inhibitor (Inh) complexes (Supplementary Figure S1A). Polymicrobial CS accelerated clotting in NPP in a dose-dependent manner (Supplementary Figure S1B) and was unaffected by the addition of vehicle alone (Supplementary Figure S1A, B). Higher concentrations of CS produced these same results but also induced activation of FXII to FXIIa *ex vivo* (Supplementary Figure S1C).

3.4 | Pharmacological inhibition of HK limits contact system activation and clotting induced by polymicrobial CS in human plasma

HK is a pivotal protein involved in regulating activation of both arms of the plasma contact system [23–25,33]. We have previously shown that the 3E8 anti-HK antibody [34] can prevent contact system

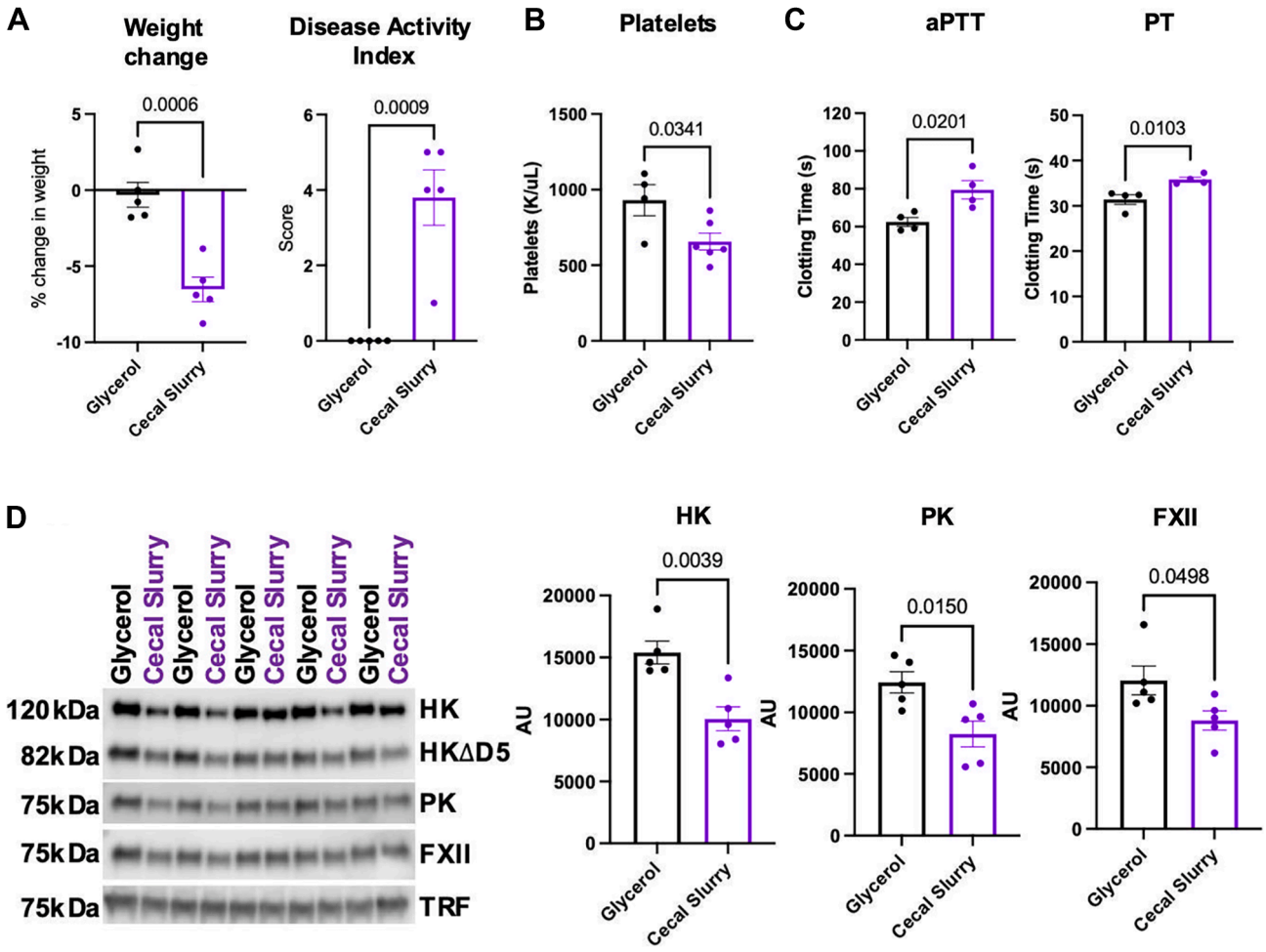

FIGURE 2 Cecal slurry (CS) model of polymicrobial sepsis activates the plasma contact system *in vivo*. CS or glycerol (vehicle) was injected intraperitoneally into recipient wild-type mice. (A) Mice administered CS exhibited symptoms of sepsis, including weight loss (left) and increased disease activity index scores (right). $n = 5$ per group; unpaired 2-tailed *t*-test. (B) CS mice had reduced circulating platelet counts as measured in a complete blood count panel. $n = 4$ to 5, unpaired 2-tailed *t*-test. (C) Plasma collected from mice 24 hours after intraperitoneal injection of CS showed delayed clotting by activated partial thromboplastin time (APTT) and prothrombin time (PT) assays. $n = 4$ per group; unpaired 2-tailed *t*-test. (D) Western blot shows mice injected with CS exhibit increased plasma contact system activation as measured by reduced intact high-molecular-weight kininogen (HK) and HK isoform lacking domain 5(HKΔD5), prekallikrein (PK), and factor (F)XII. $n = 5$ per group; unpaired 2-tailed *t*-test. Data are shown as mean ± SEM. AU, arbitrary units; TRF, transferrin.

activation by blocking HK cleavage and BK generation, as well as by preventing the activation of FXI and PK and the reactivation of FXII in the presence of other plasma contact system activators like dextran sulfate and amyloid-beta [24]. We tested whether 3E8 could block CS-induced HK cleavage and PK activation *ex vivo*. When NPP was preincubated with 3E8 anti-HK antibody for 30 minutes prior to adding CS, we found that 3E8 protected against CS-induced HK cleavage and PK activation (Figure 3A).

We have previously shown that 3E8 anti-HK antibody delays intrinsic clotting specifically and has no effect on tissue factor-driven extrinsic clotting [30]. To determine if CS-induced clotting occurs via the intrinsic pathway, we treated NPP with 3E8 (670 nM) to block HK cleavage and activation of FXI and PK. Clotting was then induced by the addition of CS. 3E8 delayed CS-induced clotting in NPP (Figure 3B, left). As shown previously [25], 3E8 also delayed intrinsic clotting (ie, APTT; Figure 3B, middle) but had no effect on extrinsic (ie, PT) clotting (Figure 3B, right). In line with these results, HK-deficient human plasma showed a significant delay in CS-induced clotting compared with NPP (Figure 3C). Due to differences in protein homology between mouse and human HK, 3E8 cannot be used to inhibit HK in mouse plasma, so these experiments were only carried out in human samples.

3.5 | Polymicrobial CS exhibits endogenous FXIIa-like enzymatic activity

To test the role of FXII/FXIIa in CS-induced clotting, we treated plasma with CTI (2.66 μ M), an Inh of FXIIa. CTI was able to delay CS-induced clotting in both mouse plasma and NPP (Supplementary Figure S2A, B). As with HK deficiency (Figure 3C), deficiencies in FXI, FVII, and FX delayed CS-induced clotting (Supplementary

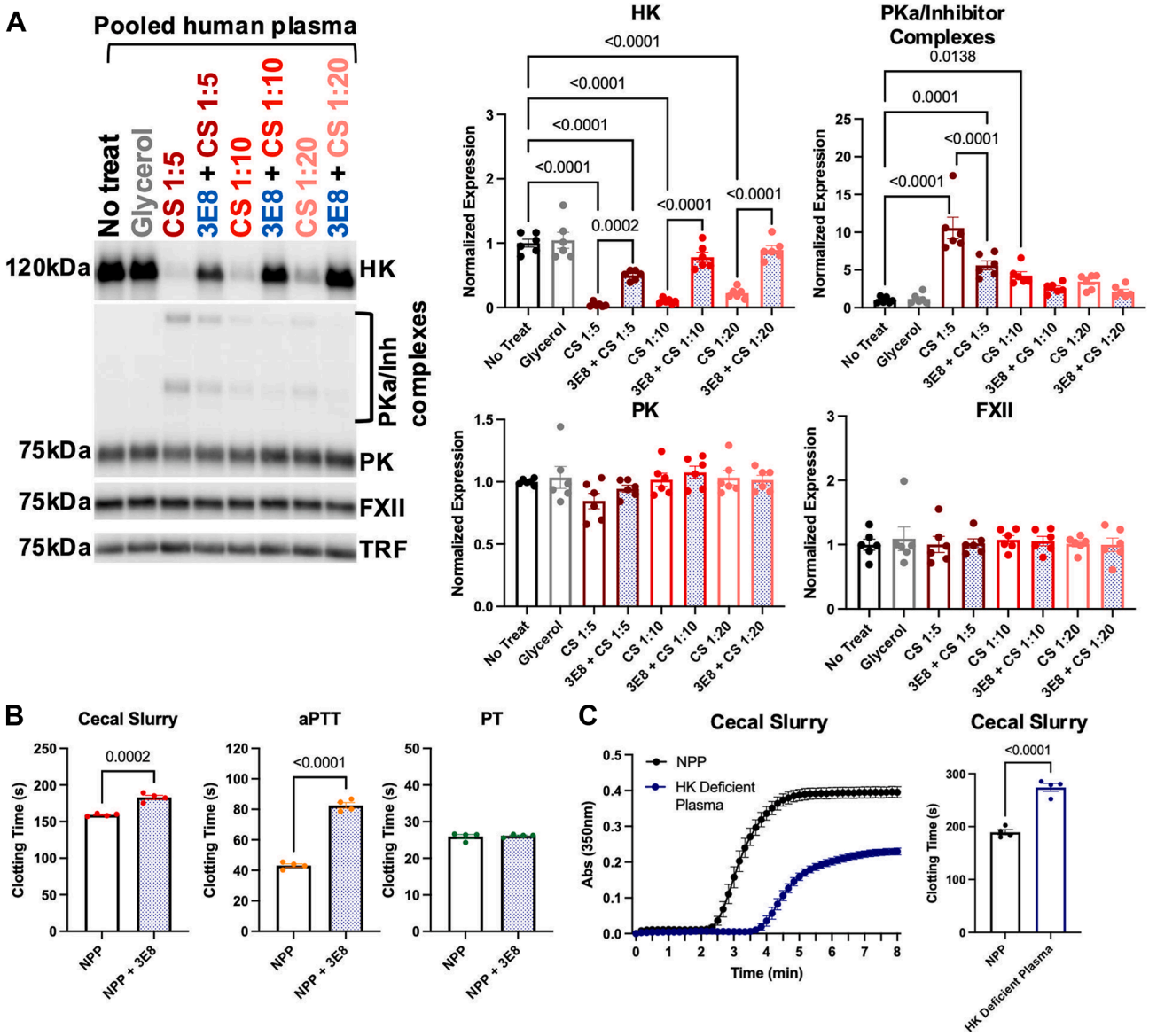

FIGURE 3 Blocking high-molecular-weight kininogen (HK) in human plasma limits contact system activation and clotting induced by polymicrobial bacteria. Normal human pooled plasma (NPP) was incubated with 3E8 anti-HK antibody (3 μ M) for 30 minutes prior to incubation with buffer (No Treat), glycerol (vehicle), or cecal slurry (CS) at the specified dilution for 1 hour at 37 °C. (A) Left, representative Western blot; right, quantification of levels of HK, prekallikrein (PK), activated PK/inhibitor (PKa/Inh) complexes, and factor (F)XII, normalized to transferrin (TRF) loading control, after incubation with CS. Results show that CS-induced contact system activation was inhibited by 3E8 anti-HK antibody (shaded blue bars). *n* = 6 per group; one-way ANOVA with Tukey's post hoc test, select statistical comparisons shown. (B) Treatment of NPP with 3E8 (670 nM) significantly delayed clotting time induced by CS (diluted 1:10 in HEPES buffer, left) and intrinsic clotting (induced by ellagic acid, middle) but had no effect on extrinsic clotting time (by prothrombin time [PT], right), as previously shown [30]. *n* = 4 per group, unpaired 2-tailed *t*-test. (C) CS-induced (diluted 1:10 in HEPES buffer) clotting was delayed in HK-deficient human plasma. Left, plot shows clotting as determined by reading absorbance (Abs) at 350 nm over time. Right, bar graph shows clotting time of NPP or HK-deficient plasma when activated by CS. *n* = 4 per group, unpaired 2-tailed *t*-test. Data are shown as mean ± SEM. kDa, kilodalton.

Figure S2C). Surprisingly, FXII-deficient plasma did not show a delay in CS-induced clotting (Supplementary Figure S2C).

These results led us to investigate whether polymicrobial CS has any endogenous FXIIa activity. When CS was incubated with chromogenic substrate S-2302 (FXIIa/PKa activity) in the absence of plasma, we detected high levels of activity, suggesting the presence of an endogenous protein with FXIIa/PKa-like activity (Figure 4A). Heat inactivation of the CS (ie, HI-CS, heated at 65 °C for 20 minutes) abrogated the FXIIa/PKa-like activity (Figure 4A). We then tested whether CS or HI-CS could activate FXIIa/PKa in plasma. NPP

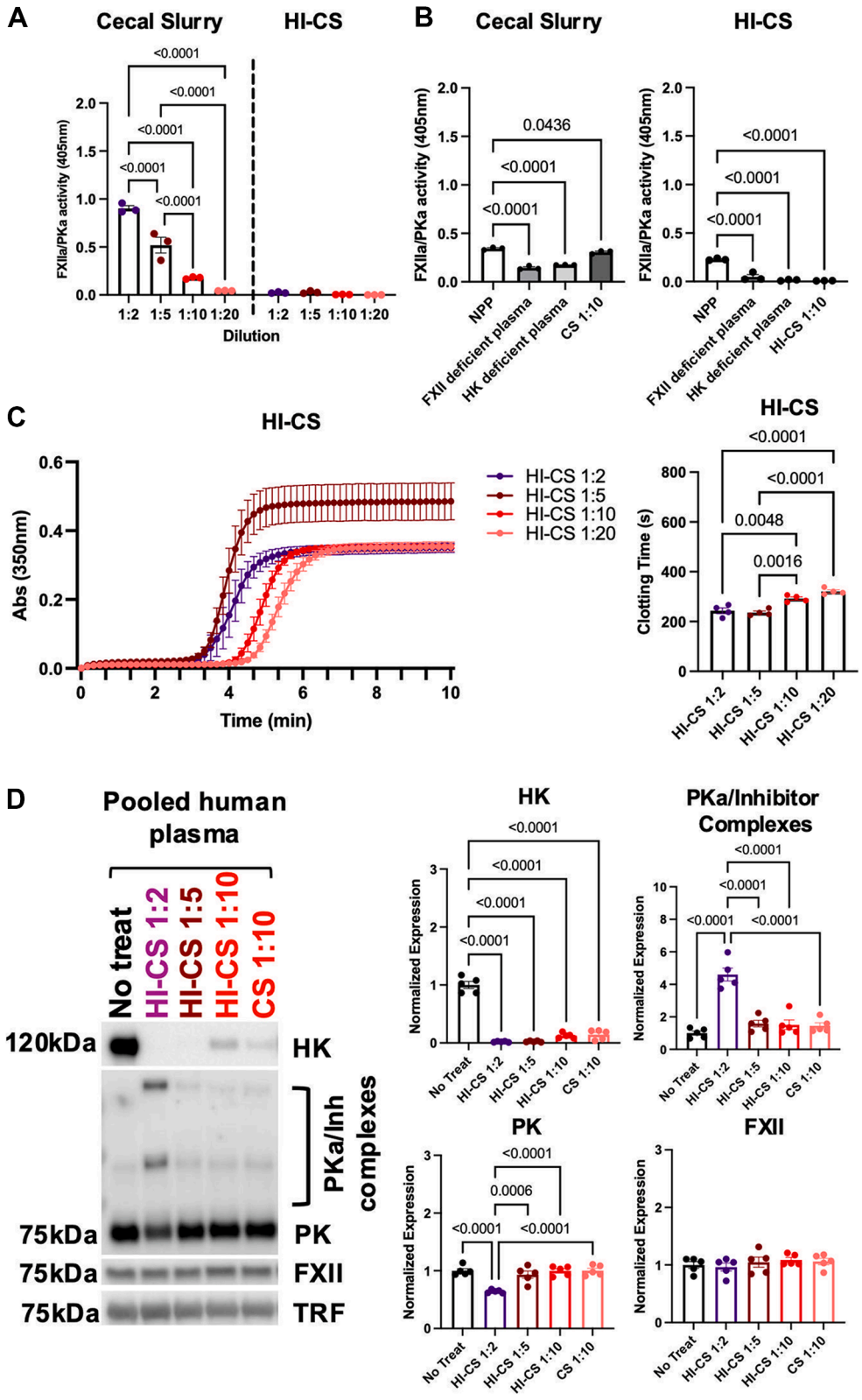

FIGURE 4 Clotting induced by heat-inactivated cecal slurry (HI-CS) in normal pooled plasma (NPP) is dependent on factor (F)XII and high-molecular-weight kininogen (HK) activity. (A, B) Activated FXII (FXIIa)/activated prekallikrein (PKa)-like activity in cecal slurry (CS) and HI-CS was measured using chromogenic substrate S-2302 (S-2302). Activity was determined as absorbance (Abs) at 405 nm. (A) Bar graph shows endogenous activity in CS (left) or HI-CS (right) when varying dilutions were incubated with S-2302 after 12 minutes. *n* = 3 per group, 2-way ANOVA with Tukey's multiple comparisons test, select comparisons shown. (B) CS (left, diluted 1:10 in HEPES buffer) or HI-CS (right, diluted 1:10 in HEPES buffer) was incubated with NPP, FXII-deficient plasma, HK-deficient plasma, or alone (no plasma), as well as with S-2302 to measure FXIIa/PKa activity after 20 minutes. Both CS and HI-CS increased FXIIa/PKa activity in NPP significantly compared with CS alone or

incubated with CS induced greater FXIIa/PKa activity than CS incubated alone (Figure 4B). In comparison, CS-induced FXIIa/PKa was significantly reduced in both FXII-deficient and HK-deficient plasmas (Figure 4B). NPP incubated with HI-CS showed significant FXIIa/PKa activation compared with HI-CS incubated with the chromogenic substrate alone (Figure 4B). As expected, FXII-deficient and HK-deficient plasmas incubated with HI-CS showed no FXIIa/PKa activity (Figure 4B). The FXIIa-like activity detected in CS was also able to cleave FXI to generate FXIa, and incubation of CS with recombinant FXI increased the detectable FXIa activity as measured by chromogenic substrate (Supplementary Figure S3). These data suggest that CS itself contains an endogenous protein that functions enzymatically similar to FXIIa that is successfully inhibited or denatured upon heat inactivation.

3.6 | HI-CS activates the plasma contact system and induces clotting in NPP

HI-CS accelerated clotting in NPP in a dose-dependent manner (Figure 4C). HI-CS–induced clotting was delayed by incubating plasma with 3E8 or CTI to inhibit FXIIa (Supplementary Figure S4A). HI-CS–induced clotting was dependent on FXII, FXI, HK, FX, and FVII, as deficiencies in any of these coagulation factors induced a delay in or an absence of clotting (Supplementary Figure S4B). Incubation of NPP with HI-CS showed a dose-dependent activation of the plasma contact system *ex vivo*. HI-CS induced the cleavage of HK, the activation of PK, and the generation of PKa/Inh complexes, as measured by Western blot (Figure 4D).

These data suggest that CS and HI-CS both induce clotting in plasma, albeit to different degrees. Pharmacological inhibition of or congenital deficiency in either FXII or HK delays clotting induced by HI-CS. Pharmacological inhibition of or congenital deficiency in HK delays clotting induced by CS.

4 | DISCUSSION

Understanding how to limit clotting and inflammation in septic conditions could provide novel therapeutic opportunities to limit the severity and societal burden associated with the disease. Here, we show that the CS model of polymicrobial sepsis can activate the plasma contact system and induce clotting. *Ex vivo* incubation of plasma with CS-induced plasma contact system activation, which activated FXII, cleaved HK, and activated PK in a dose-dependent manner. As a functional consequence, CS was also able to promote clotting in mouse plasma. These data suggest that the bacterial components in the CS interact with proteins of the plasma contact system directly, and that the resulting activation is independent of the host inflammatory response or any bacteria-mediated platelet activation.

In vivo, i.p. injection of polymicrobial CS caused mice to develop sepsis-like symptoms. Septic mice exhibited activation of the plasma contact system, as determined by reduced levels of intact plasma HK, PK, and FXII in circulation. Importantly, mice administered CS exhibited reduced circulating platelets as well as prolonged APTT and PT, as is typically observed in septic patients and murine models of sepsis [32,35–37], suggesting activation of both intrinsic and extrinsic coagulation pathways *in vivo*. This result is particularly important as prolonged APTT and PT are positively correlated with mortality in humans, suggesting that inhibiting bacteria-induced coagulation could be critical in reducing disease severity and preventing lethality in patients.

As found with mouse plasma, polymicrobial CS was able to induce contact system activation and clotting in human plasma *ex vivo*. *Ex vivo* incubation of NPP with CS resulted in dose-dependent activation of FXII, cleavage of HK, and activation of PK. Consequently, CS was able to induce clotting in NPP in a dose-dependent manner.

We have previously shown that HK is a pivotal protein in regulating activation of the plasma contact system [23–25,33]. Pharmacological inhibition of HK using our monoclonal 3E8 antibody was sufficient to reduce CS-induced HK cleavage, PK activation, and clotting in NPP *ex vivo*. Concurrently, CS-induced clotting was significantly delayed in HK-deficient plasma compared with NPP. Collectively, these data suggest that HK regulates both CS-induced contact system activation and clotting and that targeting HK with 3E8 may have therapeutic potential in limiting coagulation in septic conditions.

We further explored the role of FXII/FXIIa in regulating CS-induced clotting. Of note, other studies have highlighted conditions in which the contact system can be activated in a FXII-independent manner [14–18,38]. This result is particularly intriguing as mouse models with HK deficiency but not FXII deficiency have been shown to be protective in conditions where bacterial infection is directly or indirectly part of the disease, such as in inflammatory bowel disease and sepsis. Treatment of NPP with CTI, a FXIIa Inh, was able to delay clotting. Surprisingly, FXII-deficient plasma was not protected from CS-induced clotting (no delay in clotting time compared with NPP). This result is likely due to the presence of a protein in the CS that can function in an enzymatically similar fashion to FXIIa and can therefore activate the contact system and

HI-CS alone. This effect was ablated in FXII- and HK-deficient plasma. n = 3 per group, one-way ANOVA with Tukey's post hoc test. (C) HI-CS induced clotting in human NPP in a dose-dependent manner. Left, plot shows clotting as determined by Abs at 350 nm over time. Right, bar graph shows clotting time of pooled human plasma when activated by varying doses of CS. n = 4 per group, one-way ANOVA with Tukey's post hoc test. (D) Human NPP was incubated with buffer (No Treat), HI-CS at the designated dilutions, or CS (diluted 1:10) for 1 hour at 37 °C. Left, representative Western blot; right, quantification of levels of HK, prekallikrein (PK), PKa/inhibitor (Inh) complexes, and FXII normalized to transferrin (TRF) loading control. n = 5 per group, one-way ANOVA with Tukey's post hoc test. Data are shown as mean ± SEM. kDa, kilodalton.

the intrinsic clotting pathway in the absence of endogenous FXII. In support of this hypothesis, we found that CS itself exhibited high levels of FXIIa/PKa-like activity that cleaved recombinant FXI to generate FXIa. This activity was ablated when the CS was heat-inactivated, thereby inhibiting or degrading the proteins in the slurry responsible for this activity. Thus, the efficiency of CTI in delaying CS-induced clotting could be due to Inh effects of a protein in the CS itself. However, further work is needed to confirm this hypothesis.

Studies have shown that detrimental symptoms of sepsis can persist even after the invading pathogen has been inhibited or killed [9,39]. HI-CS was able to induce clotting in a dose-dependent manner, albeit less effectively than polymicrobial CS. The sustained ability of HI-CS to induce activation of the contact system and clotting could be due to the presence of long-chain polyphosphates, which are produced by bacteria and are known to activate FXII directly [40–43]. Pharmacological targeting of HK with 3E8 was effective in delaying clotting induced by either CS or HI-CS. Our data highlight the therapeutic potential of targeting the plasma contact system with 3E8 anti-HK antibody to limit coagulation both during active bacterial infection and in cases where symptoms persist after the pathogen is killed.

Reflective of the prolongation of APTT and PT in our *in vivo* model, FVII- and FX-deficient plasma also showed delays in CS- and HI-CS–induced clotting. This result suggests that both the extrinsic and intrinsic clotting pathways are activated in our model of polymicrobial sepsis. Specific targeting of the intrinsic coagulation pathway in sepsis may allow for a reduction in clotting while limiting the risk of bleeding.

In conclusion, our data suggest that HK could be a novel target for treatment of sepsis to reduce (1) inflammation by inhibiting HK cleavage and thus BK generation and (2) coagulation by inhibiting intrinsic clotting. This study highlights that HK is a potential target in conditions where live or dead bacteria are driving coagulation. Further, our monoclonal 3E8 anti-HK antibody was effective in limiting HK cleavage and delaying clotting by polymicrobial CS in NPP *ex vivo*. The potential for treatment of septic patients with 3E8 is promising; however, further studies are required to fully understand the efficiency and potential of anti-HK treatment *in vivo* in sepsis.

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AUTHOR CONTRIBUTIONS

A.B., E.H.N., and S.S. conceived and designed the overall study. A.B. performed *in vivo* and *ex vivo* experiments. S.J.W. performed *in vivo* experiments. M.C. performed *ex vivo* experiments. A.B. wrote the manuscript with input from all coauthors. All authors read and approved the manuscript.

DECLARATION OF COMPETING INTERESTS

The authors declare that the 3E8 anti-HK antibody is licensed to Millipore-Sigma. There are no other conflicts of interest.

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SUPPLEMENTARY MATERIAL

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