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# Factor XII activation promotes platelet consumption in the presence of bacterial-type long-chain polyphosphate *in vitro* and *in vivo*

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## Abstract

**Objective—**Terminal complications of bacterial sepsis include development of disseminated intravascular consumptive coagulopathy. Bacterial constituents, including long-chain polyphosphates (polyP), have been shown to activate the contact pathway of coagulation in plasma. Recent work shows that activation of the contact pathway in flowing whole blood promotes thrombin generation and platelet activation and consumption distal to thrombus formation *ex vivo* and *in vivo*. Here, we sought to determine whether presence of long-chain polyP or bacteria in the bloodstream promotes platelet activation and consumption in a coagulation factor (F)XII-dependent manner.

**Approach and Results—**Long-chain polyP promoted platelet P-selectin expression, microaggregate formation and platelet consumption in flowing whole blood in a contact activation

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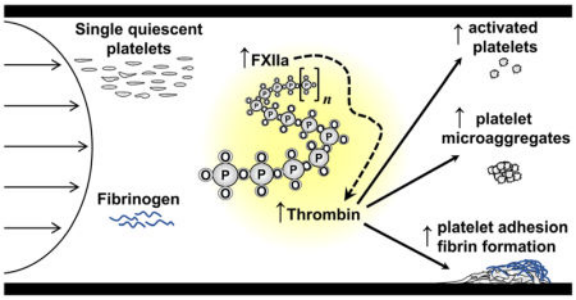
**DISCLOSURES**

A. Gruber, E.I. Tucker, and Oregon Health & Science University have a significant financial interest in Aronora, Inc., a company that may have a commercial interest in the results of this research. This potential conflict of interest has been reviewed and managed by the Oregon Health & Science University Conflict of Interest in Research Committee. S.A. Smith, R.J. Travers and J.H. Morrissey are co-investors on patent applications of medical uses of polyphosphate and polyphosphate inhibitors. D Gailani is a consultant for several pharmaceutical companies with interests in targeting factors XI and XII for therapeutic purposes. The remaining authors declare no competing financial interests.

pathway-dependent manner. Moreover, long-chain polyP promoted local fibrin formation on collagen under shear flow in a FXI-dependent manner. Distal to the site of thrombus formation, platelet consumption was dramatically enhanced in the presence of long-chain polyP in the blood flow in a FXI- and FXII-dependent manner. In a murine model, long-chain polyP promoted platelet deposition and fibrin generation in lungs in a FXII-dependent manner. Lastly, in a non-human primate model of bacterial sepsis, pretreatment of animals with an antibody blocking FXI activation by FXIIa reduced $LD_{100}$ *S. aureus*-induced platelet and fibrinogen consumption.

**Conclusions—**This study demonstrates that bacterial-type long-chain polyP promotes platelet activation in a FXII-dependent manner in flowing blood, which may contribute to sepsis-associated thrombotic processes, consumptive coagulopathy and thrombocytopenia.

## Graphic Abstract




### Keywords

sepsis; polyphosphate; coagulation; factor XII; factor XI; platelets; flow

### Subject codes

platelets; thrombosis; anticoagulants

## INTRODUCTION

Sepsis is an infection-induced systemic inflammatory response syndrome that can progress into terminal hypotension, insufficient organ perfusion and death within hours to days when left untreated.[1] Some forms of sepsis are accompanied by disseminated intravascular coagulation (DIC), a thrombo-hemorrhagic condition that aggravates poor tissue perfusion by causing blood vessel occlusion and severe bleeding due to consumptive coagulopathy and hyperfibrinolysis.[2] At present, DIC, which is a prevalent cause of sepsis-associated mortality, has no disease-specific effective FDA-approved antithrombotic treatment. There is a clear need for a better understanding of the molecular basis of sepsis/SIRS in order to develop safe and effective therapies to improve clinical outcomes.

Several lines of evidence suggest that components of the contact activation pathway of coagulation influence the host response to infection by triggering thrombin generation.[3] It has been proposed that during sepsis, exposure of blood to foreign materials, including components of pathogens (e.g., long-chain polyphosphates (polyP)), leads to FXII

autoactivation and the reciprocal activation of prekallikrein (PK) and FXII, which is accelerated in the presence of high molecular weight kininogen (HK).[4] FXIIa promotes coagulation by activating FXI, termed the intrinsic pathway of coagulation. The resulting thrombin generation drives platelet activation and fibrin formation, which may contribute to the innate immune response to select pathogens. However, with escalating intravascular coagulation, the endogenous regulators of coagulation are eventually overwhelmed, leading to vessel occlusion, hypoperfusion, and organ damage.[5] Hemostatic blood components are consumed in the process, and a hemorrhagic disorder is superimposed on the thrombotic condition.

Epidemiological data show that FXI deficiency in humans is thromboprotective,[6,7] while experimental induction of contact activation of coagulation *in vivo* can imitate thrombohemorrhagic and other sepsis-associated complications.[8] Specifically, FXI activation by FXIIa has been shown to promote thrombin generation and contribute to local platelet activation and consumption of platelets in the bloodstream distal to sites of thrombus formation under shear flow *ex vivo* and *in vivo*.[9,10] Conversely, inhibition of FXI activation by FXIIa has been shown to attenuate coagulopathy development and promote survival in mice with polymicrobial sepsis[11] as well as prevent collagen-coated vascular graft occlusion in a primate model of thrombosis.[12] It is unclear whether bacterial components such as long-chain polyP are capable of promoting systemic thrombin generation in the circulating blood.

Inorganic polyP molecules consist of tens to thousands of negatively charged phosphate groups which can be introduced into the bloodstream by activated platelets (short-chain polyP, ~70–100 phosphate units)[13] or a variety of microorganisms (long-chain polyP).[14] Synthesized or purified polyP molecules from bacteria or platelets are able to promote fibrin formation by directly supporting FXII autoactivation and enhancing activation of FV by either thrombin or FXIa,[15,16] with higher potency at increased polyP polymer lengths.[17] Long-chain polyP has also been shown to accelerate FXIa activation by both FXIIa and thrombin as well as prothrombin activation by FXIIa independent of FXI.[18,19]

In this study, we used open and closed systems to determine whether long-chain polyP is capable of promoting platelet consumption and coagulopathy in flowing blood. We employed the FXIIa inhibitor corn trypsin inhibitor (CTI), a novel anti-FXIIa-blocking antibody, 5C12, and three monoclonal anti-FXI antibodies: 14E11, which blocks FXI activation by FXIIa; 1A6, which blocks both FXI activation by FXIIa and FXIa activation of FIX, and 10C9, which is a FXIa active site domain-neutralizing antibody. Our results demonstrate that long-chain polyP promotes platelet deposition, activation, aggregation and consumption in the bloodstream in a FXII-dependent manner *in vitro* and *in vivo*. Furthermore, in a non-human primate model of bacterial sepsis, pretreatment of animals with humanized 14E11 to block FXI activation by FXIIa diminishes $LD_{100}$ *S. aureus*-induced platelet and fibrinogen consumption. These results suggest that neutralization of FXII activation in the bloodstream may represent a useful approach to preventing coagulopathy in conditions where significant levels of bacteria-type long-chain polyP are present.

## MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author on request. Details of the major resources can be found in the online-only Data Supplement.

### Reagents

Anti-FXI function-blocking antibodies 1A6, 14E11 and 10C9 were generated as described.[8,19,20] CTI was from Enzyme Research Laboratories, Inc., hirudin was from Hyphen Biomed, D-Phe-Pro-Arg-chloromethylketone (PPACK) was from Santa Cruz, fibrillar collagen was from Chrono-Log Corp, lipidated tissue factor (TF; Innovin® PT reagent) was from Siemens, and ellagic acid was from Pacific Haemostasis. Rabbit anti-fibrinogen antibody was from Cappel MP Biomedicals, LLC, anti-rabbit-AF350 was from Life Technologies, anti-CD41-PE and anti-CD62P-APC were from BD Pharmingen, and CD31-eFluor450 was from eBioscience. Anti-thrombin API (H-85) antibody was from Santa Cruz Biotech, and anti-integrin αIIb was from Abnova. Plasma-derived FXII, PK and HK were from Enzyme Research Laboratories, Inc. (South, IN, USA). FXIIa chromogenic substrate S-2302 was purchased from Diapharma. Polyphosphate molecules of the size produced by bacteria (long-chain polyP, >595 phosphate units in length) or platelets (short-chain polyP, ~70–100 phosphate units in length) were prepared as described.[17] PolyP concentrations are reported in terms of phosphate monomer concentration (monomer formula: $NaPO_3$). Recombinant polyP-binding domain from *Escherichia coli* exopolyphosphatase (PPXbd) was produced as described.[18] All other reagents were from Sigma-Aldrich, Inc. or previously named sources.[9]

### Development of an anti-factor XII function-blocking antibody

The function-blocking anti-factor XII antibody 5C12 was produced using a similar approach as described previously.[21] In brief, the murine FXII null genotype (C57Bl/6 background)[22] was crossed onto the BALB/c background through 7 generations. FXII-deficient Balb-C mice were given 25 μg of a mixture of human and murine FXII and FXIIa by intraperitoneal injection in Freund complete adjuvant on day 0 and Freund incomplete adjuvant on day 28. Hybridomas were screened using a solid phase enzyme-linked immunosorbent assay (ELISA) against human FXII, and those that showed binding were subcloned twice by limiting dilution. The clone 5C12 was chosen based on the ability of the antibody to prolong the activated partial thromboplastin clotting time of normal human plasma. The cell line producing 5C12 was grown in a CL1000 bioreactor (Integra Biosciences), and the antibody was purified from the media using cation exchange and protein A chromatography.

### Humanization of a function-blocking anti-factor XI antibody

The murine monoclonal antibody 14E11 was generated by immunizing FXI-deficient BALB/c mice with recombinant mouse FXI. 14E11 was selected for further study based on its ability to prolong the aPTT in mammalian plasmas. 14E11 exerts its anticoagulant activity in part by binding to the FXI A2 domain and preventing assembly of the contact activation complex composed of HK, PK, and FXIIa, which inhibits FXI activation by FXIIa as well as the ability of FXIa to activate FXII and the autoactivation of FXIa by polyanions.[15] The humanized form of this antibody (h14E11) was produced by complementarity

determining region (CDR)-grafting from the precursor murine monoclonal antibody 14E11. Subsequently, h14E11 was manufactured using fed-batch fermentation of h14E11-expressing CHO DUKX B11 cells in a 1000 L bioreactor, followed by protein A affinity purification, viral inactivation at low pH, and two polishing chromatography steps, followed by nanofiltration and formulation.

### Enzyme-linked immunosorbent assay to quantify FXI(a) binding to (h)14E11

FXI or FXIa (2 μg/ml, 100 μl/well) in 50 mM $Na_2CO_3$ pH 9.6 were incubated overnight at 4°C in Immulon® 2HB microtiter plates. Wells were blocked with 150 ml phosphate buffered saline (PBS) with 2% BSA for one hr at RT. Increasing concentrations of biotinylated 14E11 or h14E11 in 90 mM HBS (HEPES pH 7.2, 100 mM NaCl, 0.1% BSA, 0.1% Tween-20) was added to each well and incubated for 90 min at RT. After washing with PBST (PBS-0.1% Tween-20), 100 ml strepavidin-HRP (1:8000 dilution in HBS) was added and incubated at RT for 90 min. After washing, 100 ml Substrate Solution (30 mM citric acid, 100 mM $Na_2HPO_4$ pH 5.0, 1 tablet OPD, 30% $H_2O_2$) was added. Reactions were stopped after 10 min with 2.5M $H_2SO_4$. Absorbance at 495 nm was measured on a SpectroMax 340 microplate reader. Apparent $K_d$ values were calculated by plotting the dose-response data against the $\log_{10}$ of the antibody concentration and fitting data to a four-parameter logistic curve. The apparent $K_d$ values of h14E11 for human FXI and FXIa are 3.66 nM and 1.38 nM, respectively; the apparent $K_d$ values of 14E11 for human FXI and FXIa are 0.43 nM and 0.15 nM, respectively.

### Blood collection and preparation of plasmas

Human venous blood was drawn by venipuncture from healthy male and female adult volunteers into sodium citrate (in 0.32% w/v sodium citrate unless otherwise noted) in accordance with the OHSU Institutional Review Board (IRB #1673). Informed consent was received from all human blood donors. Platelet-poor plasma (PPP) was prepared by centrifugation of citrated whole blood from three separate donors at 2150×*g* for 10 min. Further centrifugation of the plasma fractions at 2150×*g* for 10 min yielded PPP, which was pooled and stored at −80°C until use.

### Fibrin generation assay

Solutions containing human PPP were incubated with vehicle or long-chain polyP (33, 100 or 300 μM) at 37°C for 10 min. Parallel reactions were performed in the presence of FXI function-blocking antibodies 1A6 (50 μg/mL) or 14E11 (50 μg/mL), the FXII inhibitor CTI (40 μg/mL), the thrombin inhibitor hirudin (40 μg/mL), or PPXbd (250 μg/mL). Fibrin generation was initiated with addition of either 25 mM $CaCl_2$ alone or together with 100 pM human α-thrombin. Changes in solution turbidity (A405) in clear flat-bottom polystyrene wells at 37°C were quantified using a Tecan microplate reader. The concentration of PPP was maintained at 33% final v/v in all conditions. Long-chain polyP was diluted in buffer containing 25 mM Hepes and 150 mM NaCl with 1% BSA (pH 7.4). The time interval required for the solution turbidity to reach the half-maximal value was defined as $T_{half\text{-}max}$ as described previously.[23]

### Clotting time assay

Clotting times of PPP or whole blood were measured with a KC4 Coagulation Analyzer. Samples were pretreated with FXI function-blocking antibodies 1A6 (50 μg/mL final) or 14E11 (50 μg/mL), the FXII inhibitor CTI (40 μg/mL), the FXII function-blocking antibody 5C12 (50 μg/mL), the thrombin inhibitor hirudin (40 μg/mL), or PPXbd (250 μg/mL) and incubated with vehicle or increasing concentrations of long-chain polyP (5–300 μM) at 37°C for 3 min. Clotting was initiated with addition of either 25 mM $CaCl_2$ alone or together with 100 pM human α-thrombin at 37°C, and clotting time was recorded. The concentration of PPP and whole blood was maintained at 33% and 66% final v/v, respectively, in all conditions.

### Effects of 5C12 and CTI on FXIIa activity

The effect of anti-factor FXIIa reagents was measured as described previously.[19,21] In short, after pre-incubation with either control buffer or increasing concentrations of 5C12 or CTI for 10 min at RT, human purified FXII (50 nM) was incubated with HK (12.5 nM), PK (12.5 nM), and long-chain polyP (10 μM). After 1 hr, samples were removed and quenched with polybrene (6 μg/ml fina) to neutralize long-chain polyP and with soybean trypsin inhibitor (50 μg/ml final) to inactivate kallikrein. FXIIa activity was quantified by measuring rates of chromogenic substrate S-2302 (500 μM) conversion by FXIIa at 405 nm. Rates of S-2302 FXIIa hydrolysis were converted to FXIIa concentrations using a standard curve.

### Ex vivo flow experiments

Glass capillary tubes (0.2×2×200 mm; VitroCom) were coated with 150 μg/ml fibrillary collagen as described previously.[9] Surfaces were blocked with 5 mg/mL denatured bovine serum albumin (BSA) for 1 hr prior to assembly into a flow system as shown in Figure 1A & Figure 3A. Sodium citrate (0.38% w/v) anticoagulated whole blood was re-calcified and perfused through the chamber for up to 10 min at an initial wall shear rate of 300 $s^{-1}$. Downstream samples were collected directly into 100 μM PPACK and 1.5% w/v sodium citrate (1 tube/min of perfusion) to 50% final dilution and evaluated using flow cytometry.

### Microscopy

After blood perfusion, glass capillaries were washed with PBS and Hepes/Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 0.1% BSA; pH 7.4) containing 1.5% sodium citrate and 100 μM PPACK, followed by incubation with blocking buffer (1% BSA, 1% FCS in Hepes/Tyrode buffer) for 30 min. Glass capillaries were incubated with rabbit anti-serum against human fibrinogen (1:100) for 10 min and washed with PBS followed by an incubation with CD41-PE (1:50), CD62-FITC (1:50), and goat anti-rabbit Alexa Fluor 350 (1:500) in the dark for 10 min. Glass capillaries were washed with PBS, fixed with paraformaldehyde (PFA, 4%), and sealed with mounting media. Samples were analyzed on a Zeiss Axio Imager 2 microscope as previously described.[16]

### Western blots

Thrombi formed on collagen/TF were lysed for 5 min with 1× lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 2 mM PMSF and 10 U/ml DNase I) at

4°C, followed by treatment with 1 μM plasmin for 40 min at RT. Local fibrin deposition was evaluated by separating combined eluate samples on non-reducing SDS–PAGE gels, transferring to PVDF membrane and immunoblotting with rabbit anti-fibrinogen followed by anti-rabbit-HRP. Local platelet deposition and thrombin content was similarly evaluated by separating combined eluate samples on separate reducing SDS–PAGE gels and immunoblotting for CD41 or thrombin. Proteins were detected using ECL.

### Flow cytometry analysis

Pre- and post-chamber blood samples were collected into 100 μM PPACK and 1.5% w/v Na-citrate (1:1, v/v) and incubated with 1:50 dilution antibodies for 30 min at RT. Reactions were fixed by diluting 1:10 with 12.5% Cytofix$^{BD}$. 10,000 single platelets were determined by a PE-conjugated platelet marker CD41a and the characteristic forward- and side-scatter scatter patterns via flow cytometry. Platelet CD62P expression levels and single platelet consumption were determined as described previously.[9,10] Microaggregate formation was determined by the upshift in fluorescence intensity in CD31/CD41a double-positive events.

### Mouse model of polyphosphate-induced pulmonary embolism

Animal work was authorized and approved by the Case Western Reserve University IACUC review board. A stock concentration of 0.869 M long-chain polyP in 20 mM Hepes, 5 mM EDTA, pH 7.4 was diluted 1:10 in the same buffer and 100 μl of 0.0869 M polyP was slowly injected over 10 min into the inferior vena cava of male or female wild-type (WT), *f12*$^{-/-}$, *klkb1*$^{-/-}$, or *kgn1*$^{-/-}$ mice anesthetized with a constant flow of isoflurane. When the animal expired, usually within 2 to 15 min after injection, the lung was exposed and the trachea dissected and infused with ~1 ml of optimal cutting temperature compound (OCT) in both lungs. Afterwards, one lung was harvested and fixed in 4% paraformaldehyde. The other lung was frozen into OCT and preserved at −80°C. Tissues in paraformaldehyde were cut into 10 μm sections and stained with hematoxylin and eosin (H&E). The H&E slides were scanned at 40X on a Leica 400 Slide Scanner equipped with a Hamamatsu line sensor color camera and a 40×/0.65 NA objective and reviewed by digital microscope at 10×. The number of vessel occlusions per 10× high powered field (hpf) was determined from the H&E staining. No vessel greater than 5 cm in any dimension was included in the analysis.

### Immunofluorescent studies

Frozen lungs in OCT from the long-chain polyP infusion studies were cut into 10 μm sections. Lung frozen sections were removed from the freezer and air dried for 10–15 min at room temperature. After blocking with 5% fetal bovine serum in PBS for 1 hr, sections were rinsed. All primary antibodies were used at 1 μg/ml in solutions in PBS containing 1% fetal bovine serum. The presence of murine fibrin in the lungs infusion was determined by incubation for 1 hr with mAb 59D8 (generously provided by H. Weiler, Blood Center Wisconsin), followed by incubation for 1 hr with a goat-anti-mouse antibody conjugated with Alexa Fluor 488 in PBS. Platelet antigen in the same lung sections was determined on the same slides using a rat anti-mouse mAb to GPIbα; Emfret Analytics) followed by incubation for 1 hr with a donkey anti-rat antibody (1:8000 dilution) conjugated with Alexa Fluor 594 (Invitrogen). The coverslips were mounted and fixed with ProLongTM Diamond Antifade Mountant with DAPI (Invitrogen). Photomicrographs of the lung tissue were

viewed on a Leica DM6000 upright microscope at 10× or 20× final magnification. In other experiments, the constitutive expression of CD31 in lungs for each genotype was determined. Frozen sections prepared as above were incubated for 1 hr with a rat anti-mouse CD31 (Santa Cruz) antibody followed by 1 hr of a donkey anti-rat antibody conjugated with Alex Fluor 594. The number of vessels was determined by CD31 expression in the lung tissues as determined by pixels/unit of area/10× hpf via ImageJ analysis (NIH).

### Non-human primate model of sepsis

Animal work was authorized and approved by the Oklahoma Medical Research Foundation and University of the Free State IACUC review boards. Male or female *Papio ursinus* baboons were dosed with a single 1 mg/kg intravenous bolus injection of anti-FXI antibody h14E11, 30 min before challenge. A lethal dose ($1–2 \times 10^{10}$ cfu/kg) of heat-inactivated *S. aureus* (strain B17266 Rosenbach, ATCC #49496) in 1.5 mL/kg sterile saline solution was infused intravenously over 2 hrs (from $T_0$ to $T_{120}$ min). Blood samples were obtained at 0, 2, 4, 6 and 8 hrs. Platelet levels were determined using a VetScan HM5 Hematology Analyzer. Whole blood samples were spun down to isolate plasma, and plasma levels of fibrinogen levels were measured using a Clauss functional clotting assay.

### Data analysis

Data are shown as means ± SEM. Statistical significance of differences between means was determined by ANOVA. For comparing the area under the mean in Figures 1, 3, and 4, we ran the Shapiro-Wilk normality test on each treatment within each group. If any treatment within a group failed the normality test ($p < 0.05$), we analyzed that group using the non-parametric Kruskal-Wallis test with post-hoc testing by Dunn's test. Groups in which all treatments passed the normality test were analyzed with one-way ANOVA with post-hoc testing by the Tukey test. Probability values of $P < 0.05$ were selected to be statistically significant. Apparent $K_d$ values were calculated by plotting the dose response data against the $\log_{10}$ of the antibody concentration. The data was then fit to a four-parameter logistic curve and the Kd was calculated using GraphPad Prism.

## RESULTS

### Long-chain polyP promotes platelet consumption in flowing blood

Our initial set of experiments examined whether soluble long-chain polyP promotes platelet activation in flowing blood. As shown in Figure 1A, re-calcified citrated whole blood was perfused through an inert (BSA-coated) flow chamber and tubing for a total residence time of 30 seconds before being collected downstream at 1 min intervals into tubes containing a serine protease inhibitor (PPACK) and sodium citrate. Single platelet consumption (reduction of the single platelet population in the bloodstream as quantitated on a forward vs side-scatter FACS plot), platelet activation (CD62P expression), and platelet microaggregate formation in solution (CD41a/CD31 high positive) was quantified using flow cytometry as described previously.[9,10]

Our results show that addition of 100 μM long-chain polyP to whole blood resulted in a robust increase in platelet activation (>30% CD62P expression), microaggregate formation

and single platelet consumption (>80%) in the blood flow system coated with BSA, while platelets remained quiescent under vehicle control conditions (Figure 1B). We next pretreated blood with recombinant exopolyphosphatase binding domain (PPXbd), which competitively inhibits polyphosphate binding, to validate a role for long-chain polyP in promoting platelet activation in the flowing blood. Our results show that the presence of PPXbd completely neutralized the effect of long-chain polyP to control levels of platelet activation, aggregate formation and consumption (Figure 1B and Supplemental Figure I). In contrast, pretreatment of blood with short-chain polyP of the size secreted by platelets or PPXbd alone did not affect the degree of platelet activation, aggregate formation or consumption, in line with the fact that platelet-derived short-chain polyP is only a weak activator of the contact activation pathway.[19]

Platelet activation, aggregate formation and consumption in flowing blood induced by long-chain polyP was abrogated by treatment of whole blood with either a FXIa active site domain-neutralizing antibody, 10C9, the anti-FXI antibody 1A6, which inhibits FXI activation by FXIIa and FIX activation by FXIa, the anti-FXI antibody 14E11, which inhibits FXI activation by FXIIa and conversely activation of FXII by FXIa, the FXIIa inhibitor corn trypsin inhibitor CTI, or the direct thrombin inhibitor, hirudin (Figure 1C and Supplemental Figure I).

As a comparator, we used ellagic acid, a known activator of the contact pathway or tissue factor (TF), the extrinsic pathway activator, in this system to validate the role of activation of FXII or FVII, respectively, in promoting platelet consumption in flowing blood (Figure 1D and 1E and Supplemental Figure I). Our results show that ellagic acid promoted platelet activation, aggregation and consumption in flowing blood, which was eliminated in the presence of 1A6, CTI, or hirudin. The ability of TF to promote platelet activation, aggregate formation or consumption was eliminated by hirudin, while aggregate formation was insensitive to inhibition of FXI activation with 1A6 or inhibition of FXIIa with CTI, and platelet consumption was reduced by 1A6 but insensitive to CTI. Taken together, our data indicate that long-chain polyP and a known activator of the contact pathway, ellagic acid, potentiate platelet activation and consumption in flowing blood in a contact activation pathway-dependent manner.

## Long-chain polyP promotes local thrombus formation on collagen surfaces

We next studied whether long-chain polyP had an effect on local platelet deposition and fibrin formation on collagen under shear flow conditions. Citrated whole blood was incubated with either vehicle or long-chain polyP prior to re-calcification and perfusion over collagen-coated surfaces. Under vehicle conditions, we observed a time-dependent increase in the degree of platelet adhesion, aggregation, activation and fibrin formation on collagen (Figure 2A and 2B). The extent of fibrin formation was enhanced in the presence of soluble long-chain polyP, as visualized using differential interference contrast and fluorescence microscopy (Figure 2C and 2D). Long-chain polyP decreased the time required before detectable levels of d-dimer, clot-bound thrombin and platelets were observed in the formed thrombi as measured by Western blot (Figure 2E and 2F). Blockade of FXI activation with 1A6 or inhibition of FXIIa activity with CTI nearly eliminated fibrin formation both in the

presence or absence of long-chain polyP, while the degree of platelet deposition as measured by CD41a via Western blot remained fairly equivalent under all conditions. Along these lines, in the absence of coagulation, platelet adhesion and aggregation on either collagen or immobilized von Willebrand factor (VWF) under flow was unaffected by the presence of long-chain polyP (Supplemental Figure II). Conversely, in a closed system, we found that long-chain polyP promoted fibrin formation in a dose-dependent manner, decreasing the time required to reach half maximal fibrin levels ($T_{half\text{-}max}$) from 2140s ± 346s under vehicle conditions to 1332s ± 236s, 854s ± 138s and 763s ± 95s in the presence of 33, 100 and 300 μM long-chain polyP, respectively (Supplemental Figure III). The effect of long-chain polyP on fibrin formation was reversed by CTI or PPXbd. Taken together, our results suggest that long-chain polyP promotes thrombin generation to enhance local fibrin formation at sites of thrombus formation in a contact activation pathway-dependent manner.

## Long-chain polyP promotes distal platelet activation and consumption in flowing blood downstream of sites of local thrombus formation

Our previous work identified a role for FXI in promoting distal platelet activation downstream of sites of local thrombus formation.[9] We next designed experiments to determine whether the presence of long-chain polyP would increase the rate and extent of downstream platelet activation in a FXII-dependent manner. As shown in Figure 3A, the outflowing blood was collected 30 seconds downstream of a collagen-coated chamber. Our results show that platelet P-selectin expression and microaggregate formation increased as a function of time in whole blood samples downstream of collagen-coated chambers, reaching a maximum of 90% single platelet consumption by 10 min of perfusion (Figure 3B). Pretreatment of blood with the anti-FXI mAbs 14E11, 1A6 or 10C9 or the FXIIa inhibitor CTI eliminated downstream platelet activation and microaggregate formation and reduced single platelet consumption (Figure 3B and Supplemental Figure 4).

The presence of long-chain polyP significantly increased the rate and extent of distal platelet activation, microaggregate formation and single platelet consumption in flowing blood downstream of the local site of thrombus formation (Figure 3C and Supplemental Figure IV). For instance, the extent of microaggregate formation increased over 3-fold in the presence of long-chain polyP after 5 min relative to baseline, while long-chain polyP decreased the time required to achieve a 95% loss of single platelets in flowing blood from 10 min to 8 min ($EC_{50}$=5 min vs $EC_{50}$=6 min, respectively). An initial inhibition of platelet activation and microaggregate formation was observed when blood was pretreated with the anti-FXI mAbs 14E11, 1A6 or 10C9, while only CTI and hirudin were able to eliminate platelet activation and microaggregate formation over the full 10 min of observation. Taken together, our results suggest that long-chain polyP promotes distal platelet activation and consumption in flowing blood downstream of sites of active thrombus formation in a contact activation-dependent manner.

## Long-chain polyP promotes distal platelet activation and consumption in flowing blood in a FXII-dependent manner

Corn trypsin inhibitor has classically been used as a FXIIa inhibitor. However, CTI has also been shown as a weak competitive inhibitor of FXIa.[24,25] Moreover, the inhibitory effect of

CTI on FXIIa activity is transient, as it forms a one-to-one complex with FXIIa or trypsin.[26] We therefore created an anti-FXII mAb, 5C12, to use as a tool to mechanistically define the role of the contact activation pathway in the ability of long-chain polyP to promote distal platelet activation and consumption. We found that 5C12 bound human FXII in plasma (Figure 4A) and blocked the ability of long-chain polyP to activate FXII in a purified system in the presence of HK and PK, as measured by quantifying FXIIa activity using a chromogenic assay (Figure 4B). In recalcified plasma, 5C12 inhibited the ability of 100 μM long-chain polyP to promote clotting to a similar degree as CTI (Figure 4C).

We next evaluated the effect of 5C12 on platelet activation induced by long-chain polyP in our flow assay. Our data showed that 5C12 eliminated the ability of long-chain polyP to promote platelet activation, microaggregate formation or consumption when whole blood was perfused either through an inert BSA-coated chamber or over a collagen-coated surface to induce a local site of thrombus formation (Figure 4D–4F and Supplemental Figure V). These results provide additional evidence that long-chain polyP promotes distal platelet activation and consumption in flowing blood and downstream of sites of active thrombus formation in a FXII-dependent manner.

## Long-chain polyP promotes platelet deposition and fibrin generation in vivo in a FXII-dependent manner

We next designed experiments to determine whether long-chain polyP induced platelet activation and fibrin formation in the bloodstream *in vivo* in experimental pulmonary embolism. A bolus of long-chain polyP was injected into a cohort of wild-type (WT), FXII-deficient ($FXII^{-/-}$), PK-deficient ($Klkb1^{-/-}$) or HK-deficient ($Kgn1^{-/-}$) mice. In all mice, pulmonary emboli were observed (Figure 5A) containing platelets and fibrin (Figures 5B and 5C). However, the degree of platelet deposition and fibrin formation observed in the lung tissue was significantly reduced for $FXII^{-/-}$ mice as compared to WT > $Klkb1^{-/-}$ > $Kgn1^{-/-}$ mice (Figure 5D). These studies indicated that presence of long-chain polyP in the bloodstream promoted platelet activation and fibrin generation *in vivo* in a FXII-dependent manner.

## Long-chain polyP-containing bacteria induce platelet and fibrinogen consumption in vivo in a FXIIa-dependent manner

In a proof-of-concept *in vivo* experiment, we next examined whether inhibition of the ability of FXIIa to activate FXI reduces consumption of platelets and fibrinogen in an established model of bacterial sepsis in baboons, *Papio ursinus* (Figure 6). In this model, animals were challenged with heat-inactivated *Staphylococcus aureus (S. aureus)*, a pathogenic bacterium known to contain long-chain polyP,[27,28] to a lethal dose ($LD_{100}$) of $1\text{-}2\times10^{10}$ colony forming units (cfu)/kg. Half an hour prior to bacterial challenge, animals were treated with an intravenous bolus injection of the humanized anti-FXI antibody 14E11, which blocks FXI activation by FXIIa (h14E11), or vehicle. Outcomes were measured as platelet count (Figure 6A) and circulating fibrinogen (Figure 6B) as percentage of baseline values. Our results show that *S. aureus* challenge induced a drop in platelet count to 40% of baseline at 8 hours; this was partially rescued to 60% when FXI activation by FXIIa was inhibited with h14E11. *S. aureus* challenge also decreased plasma fibrinogen to 40% of baseline at 8 hours, which

was rescued to 80% in the presence of the anti-FXI antibody h14E11. Taken together this pilot experiment suggests that bacteria which contain long-chain polyP promote platelet and fibrinogen consumption in the bloodstream in a contact activation-dependent manner.

## DISCUSSION

Sepsis is an infection-induced systemic inflammatory response syndrome (SIRS) that typically progresses to terminal hypotension, insufficient organ perfusion and death within hours to days when left untreated.[1] Timely and aggressive medical management, including treatment of the underlying cause or supportive care (volume/electrolyte supplementation, vasopressors, dialysis, oxygenation, and transfusion of blood products) reduces sepsis-associated mortality.[29] Yet, these measures often fail, and sepsis remains among the top leading causes of hospital mortality.[30] Thus, advanced sepsis presents a significant problem. Fulminant or rapidly-progressing disseminated intravascular coagulation (DIC) is a deadly complication of sepsis that has no disease-specific effective antithrombotic treatment. Nevertheless, most patients in the later hemorrhagic phase of DIC are given transfusions of blood components, sometimes combined with anticoagulation, in an attempt to both reduce pathologic coagulation and support the failing hemostatic system.[31] However, the presumed efficacy of systemic anticoagulation with broad-spectrum antithrombotic agents such as heparin or activated protein C (no longer marketed) that target the extrinsic, intrinsic, and common pathways of thrombin generation has not been confirmed in large clinical trials.[32] There is a clear need for a better understanding of the molecular basis of sepsis/SIRS in order to develop safe and effective therapies to improve clinical outcomes and identify druggable molecular targets and mechanisms that contribute to thrombin generation during early or advanced sepsis.

Coagulation factor (F)XII is an abundant member of the intrinsic coagulation pathway with no known role in hemostasis but mounting evidence for a role in thrombohemorrhagic complications associated with cardiopulmonary bypass and when human blood passes over artificial surfaces. Clinically, FXII-deficient patients have a prolonged activated partial thromboplastin time (aPTT) but do not suffer from abnormal bleeding and are otherwise asymptomatic.[33] Conversely, activation of FXII *in vivo* is prothrombotic.[8] Our present results suggest that FXII is activated by polyP of the size produced by bacteria (long-chain polyP) in the bloodstream, promoting platelet activation and consumption under shear. We show that inhibition of FXIIa activity neutralizes the effect of long-chain polyP on platelets in whole blood under shear and may be a viable target for preventing platelet consumption in settings where long-chain polyP levels may be increased, e.g. bacterial sepsis. Finally, in an initial proof-of-concept experiment using an established *in vivo* nonhuman primate model of bacterial sepsis, we show that inhibition of FXI activation by FXIIa inhibits platelet and fibrinogen consumption. Future studies are required to determine whether these effects are a result of the expression of long-chain polyP on the surface of bacteria or released following lysis of select bacteria in the bloodstream.

Inorganic polyP polymers have been shown to promote thrombin generation in a FXII-dependent manner. PolyP molecules ≥30 phosphates in length directly activate FXII to generate thrombin and fibrin formation, with increasing potency observed with increasing

polyP chain length.[17] Shorter length polyP of the size secreted by platelets (short-chain polyP) is a weak activator of FXII in solution, while larger membrane-associated polyP nanoparticles on the platelet surface have been shown to trigger contact system activation.[34] Moreover, platelet-derived short-chain polyP has been shown to serve as a potent cofactor for enhancing the feedback activation of FXI by thrombin, FV activation by FXIa, and inactivation of TFPI by FXIa.[35,36] This may explain the findings reported by Zhu *et al.* in which they demonstrated that treatment of whole blood with PPXbd 1) decreased fibrin formation in thrombi formed on collagen *in vitro* and 2) increases susceptibility of thrombi to lysis in the presence of tissue plasminogen activator.[37] Along these lines, treatment of wild-type mice with PPXbd has been shown to be thromboprotective, while this effect is lost in FXII-deficient mice.[38] Our study suggests that the presence of long-chain polyP-containing bacteria in the bloodstream promotes a prothrombotic and procoagulant phenotype in a contact activation-dependent manner.

Inorganic polyP chains contain high energy phosphoanhydride bonds identical to those found in adenosine triphosphate (ATP) molecules, which are hydrolyzed to fuel reactions in cells.[14,39] Notably, polyP chains have been shown to be utilized by select bacteria to generate ATP and ADP.[40] Furthermore, *Mycobacterium tuberculosis* and some other gram positive bacteria primarily depend on polyP for energy.[41–43] Hypophosphatemia is associated with sepsis severity and may represent an outcome of bacterial sequestering of phosphate for polyP production.[44] Moreover, microbes may release long-chain polyP upon cell damage or express long-chain polyP on their cell surfaces, promoting pathogenesis.[45] For instance, *Neisseria* expose as much as half of their polyP content on their cell surface, which may act as both an antiphagocytic capsule and a promoter of virulence during infection.[46] Thus, we speculate that activation of the abundant levels of FXII found in the blood may be part of a physiological response aimed at containment of infectious agents, a strategy that unfortunately seems to fail at higher bacterial loads. In line with this thought, FXII activation in the presence of long-chain polyP promotes proteolysis of high molecular weight kininogen with concomitant bradykinin release,[47] pro-inflammatory responses[48,49] and complement system activation,[50,51] all of which can wreak havoc on an organism in the absence of balanced regulation.

While inflammation and vasoregulatory abnormalities dominate the clinical presentation of severe sepsis, excessive activation of the blood coagulation cascade associated with the development of fulminant disseminated intravascular coagulation (DIC) remains a common cause of sepsis-related mortality. DIC is a thrombo-hemorrhagic complication that compromises organ perfusion and physiological response of hemostasis by causing thrombotic occlusion of blood vessels and consumptive coagulopathy, respectively. This research may aid in the identification of potential mechanisms and introduce potential new adjuncts to current therapy by which components of the contact activation pathway of coagulation influence the host response to infection by triggering thrombin generation and systemic platelet activation and consumption.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

| | |
|---|---|
| **long-chain polyP** | polyphosphates of the size produced by bacteria, >595 phosphate units |
| **FXI/FXIa** | zymogen/activated coagulation factor XI |
| **FXII/FXIIa** | zymogen/activated coagulation factor XII |
| **HK** | high molecular weight kininogen |
| **14E11** | anti-FXI antibody, inhibits FXI activation by FXIIa |
| **h14E11** | humanized 14E11 |
| **10C9** | anti-FXI antibody, neutralizes FXIa active site domain |
| **1A6** | anti-FXI antibody, inhibits FXI activation by FXIIa and FIX activation by FXIa |
| **CTI** | corn trypsin inhibitor, inhibits FXIIa |
| **5C12** | anti-FXII antibody, neutralizes FXIIa active site domain |
| **PPXbd** | recombinant polyP-binding domain from *E. coli* exopolyphosphatase |
| **PK** | prekallikrein |

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## HIGHLIGHTS

- Long-chain polyP promotes platelet activation and consumption in flowing blood.
- Long-chain polyP promotes platelet deposition and fibrin generation *in vivo* in a FXII-dependent manner.
- Inhibition of FXI activation by FXIIa diminished *S. aureus*-induced platelet and fibrinogen consumption in a non-human primate model of bacterial sepsis.

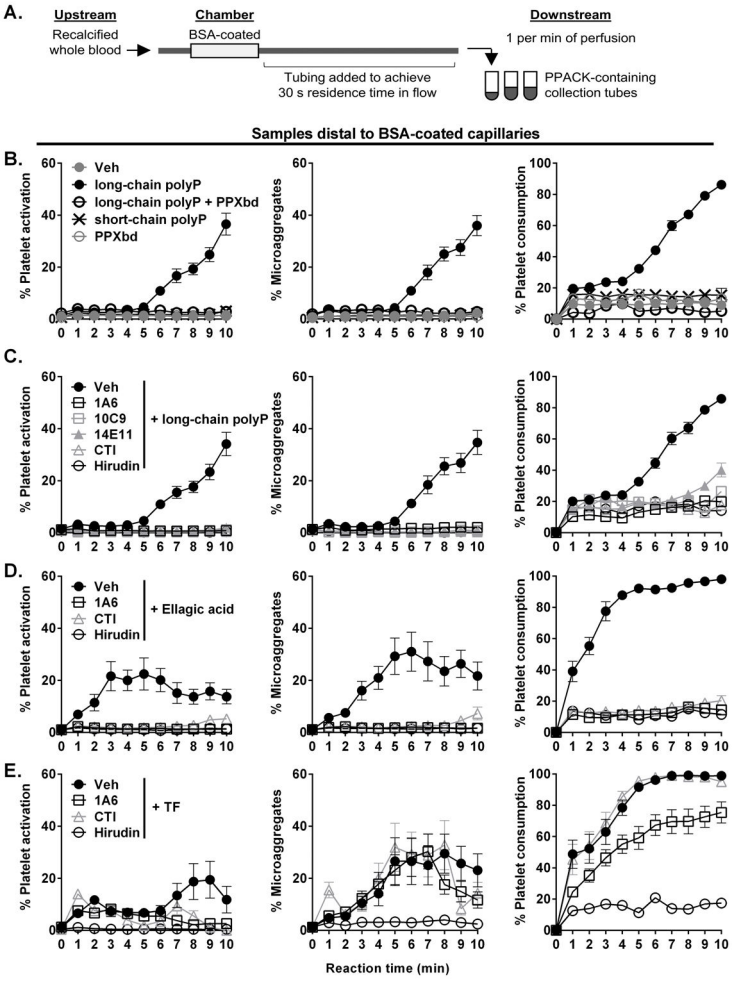


**Figure 1. Long-chain polyP promotes platelet consumption in the bloodstream**

(**A**) Re-calcified citrated whole blood was perfused through BSA-coated chambers for indicated reaction times at 300 $s^{-1}$ shear rate. Downstream samples were collected into solution containing serine protease inhibitor (100 μM PPACK) at 1 min intervals, immunostained and evaluated by FACS flow cytometry for percent platelet activation (% CD41a+/CD62P+ vs. CD41a+ total events), platelet microaggregate formation (high fluorescence intensity CD41a+/CD31+ events) and single platelet consumption (loss of single platelet population gate on FSC by SSC scatter plots). (**B**) Prior to re-calcification, blood was pretreated with either vehicle control buffer or 100 μM long- or short-chain polyP in the absence or presence of 250 μg/mL PPXbd. In separate experiments, blood was pretreated with 100 μM long-chain polyP (**C**), ellagic acid (**D**) or 100 fM tissue factor (TF; **E**) and either vehicle or anti-FXI antibodies (50 μg/mL 1A6, 10C9 or 14E11), FXIIa inhibitor (40 μg/mL CTI) or a thrombin inhibitor (40 μg/mL hirudin). Results shown as mean ± SEM from at least n>3.

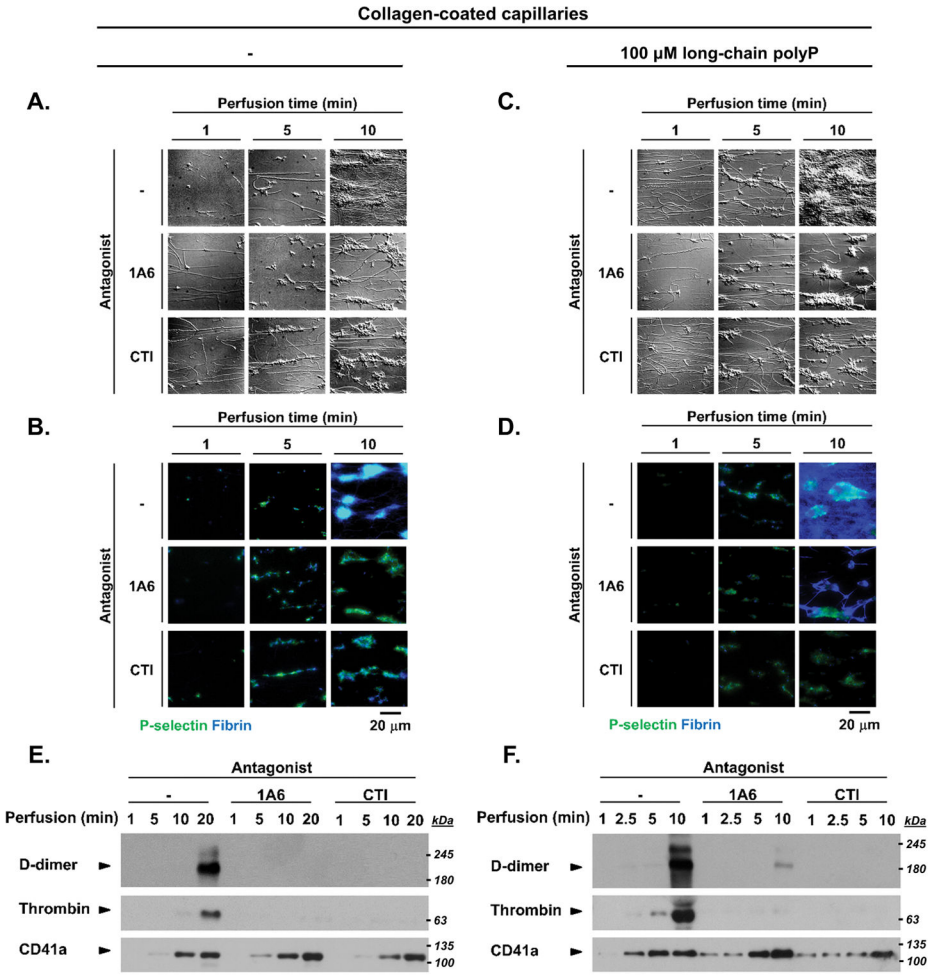


**Figure 2. The presence of long-chain polyP promotes local thrombin generation and fibrin formation on surfaces of immobilized collagen under shear flow**

Re-calcified whole blood was perfused over collagen-coated chambers for indicated perfusion times at 300 $s^{-1}$ shear rate. Images of local thrombi formed at each perfusion time point in the presence of control buffer (−), 50 μg/mL 1A6 or 40 μg/mL CTI were recorded using differential interference contrast (**A**) or fluorescent light microscopy (**B**) after staining for fibrin (blue) and P-selectin (green). In select experiments, whole blood was pretreated with 100 μM long-chain polyP prior to re-calcification and perfusion over collagen-coated chambers (**C**) and (**D**). In parallel experiments, thrombi formed in the presence of control buffer (**E**) or 100 μM long-chain polyP (**F**) by corresponding perfusion time points were subjected to detergent lysis and plasmin digest and immunoblotted for fibrin degradation product, D-dimer, thrombin and platelet surface receptor, CD41a. Representative images and blots for at least n>4.

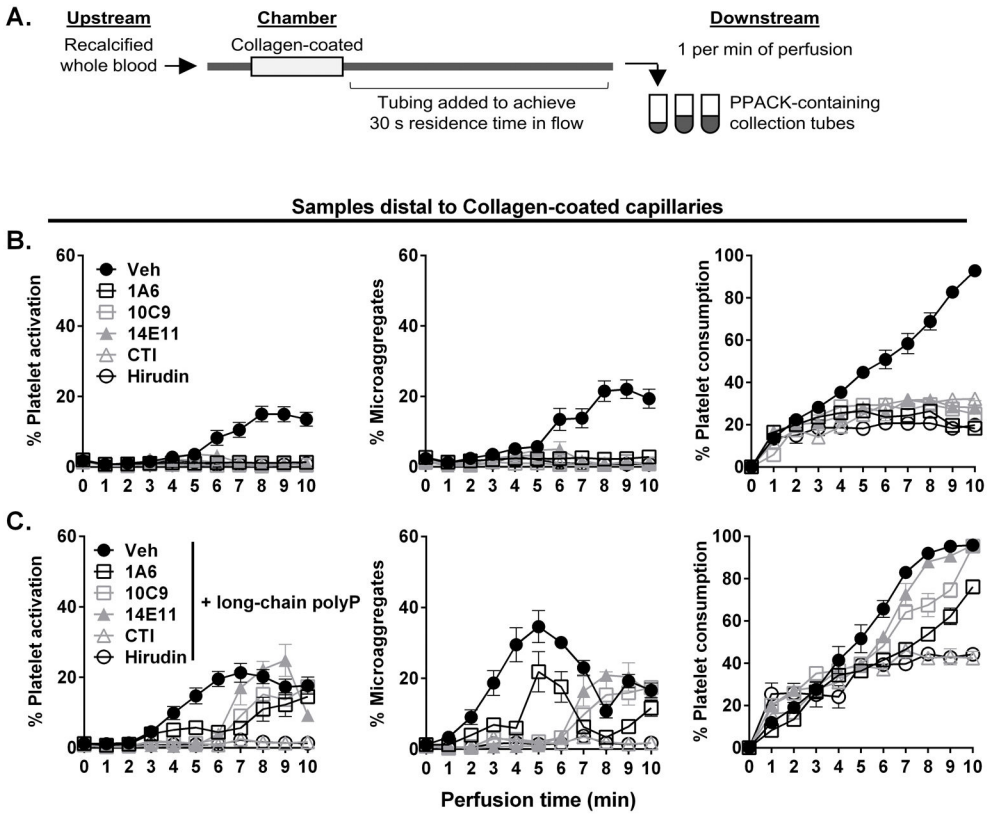


**Figure 3. Long-chain polyP promotes activation and consumption of platelets in the bloodstream distal to local thrombus formation on immobilized collagen**

Experimental set up schematic (**A**). Re-calcified whole blood was perfused over collagen-coated chambers at 300 $s^{-1}$ shear rate. Downstream samples were collected into solution containing serine protease inhibitor (100 μM PPACK) at 1 min intervals (perfusion time), immunostained and evaluated by flow cytometry (FACS). Percent platelet activation, platelet microaggregate formation and single platelet consumption in samples distal to thrombus formation on collagen-coated surfaces in the presence of vehicle control, 50 μg/mL 1A6, 10C9, 14E11, 40 μg/mL CTI or hirudin were quantified (**B**). In parallel experiments whole blood was pretreated with 100 μM long-chain polyP prior to re-calcification and perfusion over collagen-coated chambers (**C**). Representative means ± SEM for at least n>4.

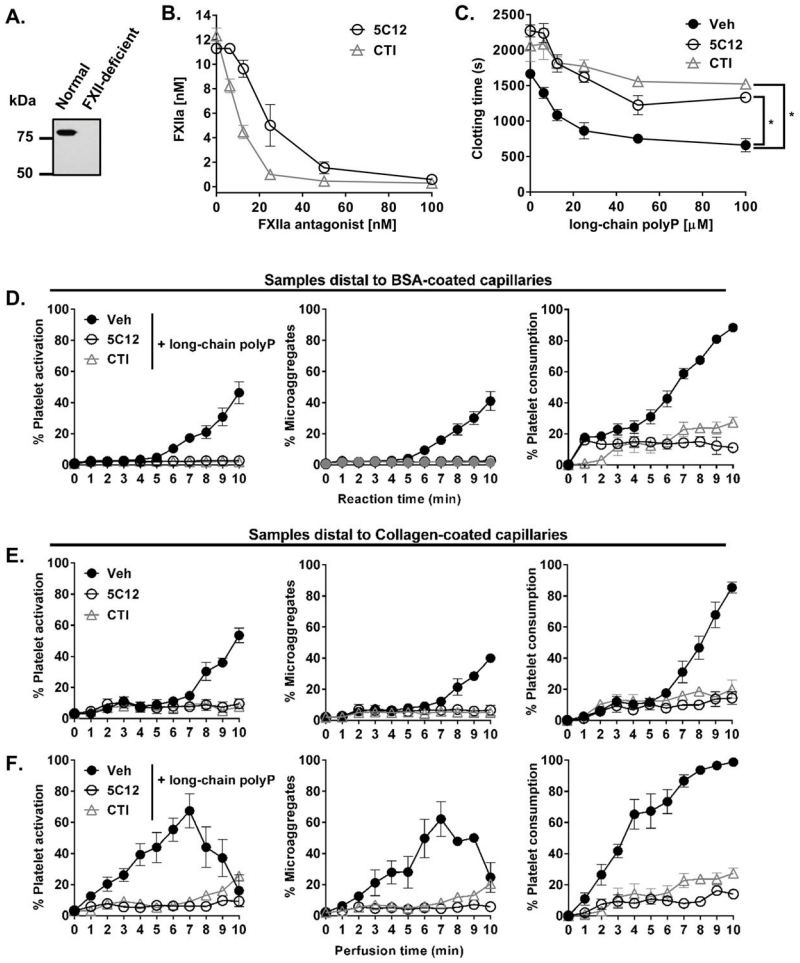


**Figure 4. Effects of an anti-FXII antibody on plasma coagulation and platelet consumption in the flowing whole human blood in the presence of long-chain polyP**

Characterization of the anti-FXII mAb, 5C12, showing that 5C12 binds to FXII in human plasma; FXII-deficient human plasma shown as a control (**A**). Human FXII was preincubated with HK, PK and long-chain polyP in the presence of increasing concentrations of 5C12 or CTI for 1 hr and the activity of FXIIa was quantified by measuring rates of chromogenic substrate S-2302. Rates of S-2302 FXIIa hydrolysis were converted to FXIIa concentrations using a standard curve (**B**). Citrated platelet poor plasma (PPP) was pretreated with vehicle, 40 μg/mL FXIIa inhibitor (CTI) or 50 μg/mL anti-FXII mAb (5C12) and incubated for 3 min at 37°C with either control buffer or 100 μM long-chain polyP prior to re-calcification to initiate clotting (**C**). Significant differences in clotting time denoted with *($p$<.05). Re-calcified whole blood was perfused through either (**D**) BSA-coated or (**E–F**) collagen-coated chambers at a shear rate of 300 $s^{-1}$. Activation, microaggregate formation and consumption of platelets was quantified in samples pretreated with either vehicle, 50 μg/mL 5C12, or 40 μg/mL CTI in the presence of vehicle control or 100 μM long-chain polyP. Means ± SEM for at least n>3.

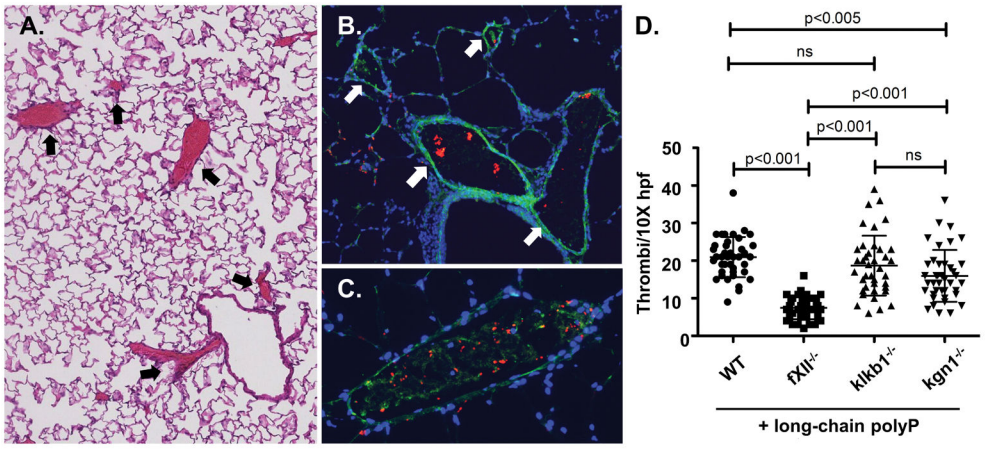


**Figure 5. Long-chain polyP promotes thrombi formation, platelet deposition and fibrin formation in lungs in a FXII-dependent manner *in vivo***

(**A**) H&E staining of normal lung sections at 10× from long-chain polyP-challenged WT mice. The black arrows point to representative occluded vessels that were used in the analysis. (**B**) Immunofluorescence staining of lung sections at 10× from long-chain polyP-challenged WT mice. Lung nuclei are DAPI-stained blue; platelets are anti-CD42b-stained red. The white arrows point to representative vessels coated with fibrin stained green. A 20× micrograph of an occluded vessel is shown in (**C**). (**D**) The number of thrombi per visual field of view was quantified in lung sections from long-chain polyP-challenged WT, *f12*$^{-/-}$, *klkb1*$^{-/-}$ and *kgn1*$^{-/-}$ mice. Shown are the raw data points, mean and standard deviation of the mean (SD) of 40 representative fields out of greater than 75 per group. Significant difference (*, $p$<.05) between the groups.

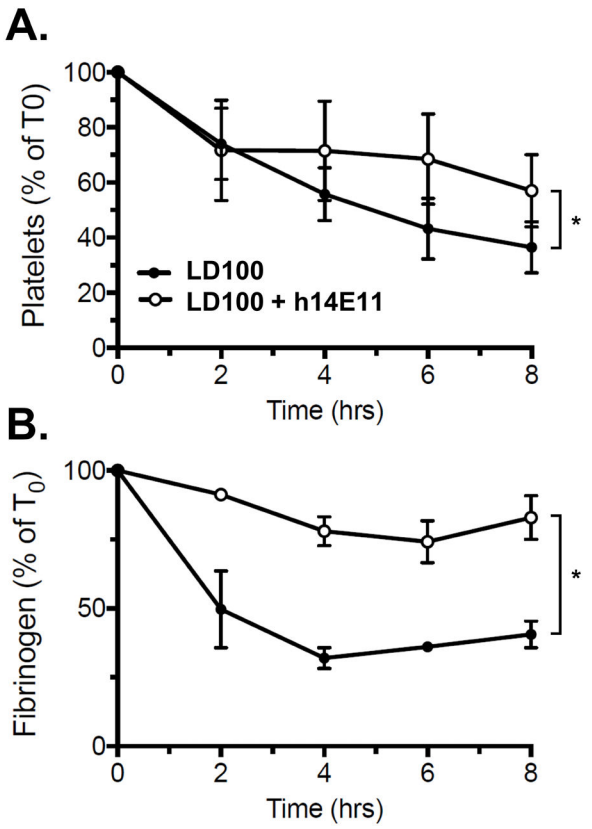


**Figure 6. Long-chain polyP-containing bacteria induce platelet and fibrinogen consumption *in vivo* in a FXIIa-dependent manner**

In a non-human primate model of sepsis, *Papio ursinus* baboons were pretreated with either normal saline or a single (1 mg/kg) bolus of anti-FXI antibody h14E11, followed by an intravenous two hour (T0 to T120 min) infusion of a lethal dose ($1–2 \times 10^{10}$ cfu/kg) of *S. aureus*. Blood samples were obtained at times 0, 2, 4, 6 and 8 hours. Platelet levels were determined using a VetScan HM5 Hematology Analyzer (**A**) and plasma levels of fibrinogen were measured using a Clauss clotting assay (**B**). Data are shown as mean ± SEM. $LD_{100}$ animals n = 2; $LD_{100}$ + h14E11 animals n = 2. Significant difference (*, $p < .05$) between the groups.