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Coagulation, an ancestral serine protease cascade, exerts a novel function in early immune defense

Running title: Factor XIII and Innate Immunity

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Abstract

Phylogenetically conserved serine protease cascades play an important role in invertebrate and vertebrate immunity. The mammalian coagulation system can be traced back some 400 million years and it shares homology with ancestral serine proteinase cascades, involved for instance in Toll receptor signaling in insects and release of antimicrobial peptides during hemolymph clotting. Here we show that induction of coagulation by bacteria leads to an immobilization and killing of *Streptococcus pyogenes* bacteria inside the clot. The entrapment is mediated via crosslinking of bacteria to fibrin fibers by the action of coagulation factor XIII (fXIII), an evolutionarily conserved transglutaminase. In a streptococcal skin infection model, $fXIII^{-/-}$ mice develop severe signs of pathologic inflammation at the local site of infection and fXIII-treatment of wildtype animals dampens bacterial dissemination during early infection. Bacterial killing and crosslinking to fibrin networks was also detected in tissue biopsies from patients with streptococcal necrotizing fasciitis supporting the concept that coagulation is part of the early innate immune system.

Introduction

Serine protease cascades play an important role in many patho-physiologic processes including hemostasis, immune response, and wound healing[1]. Their activation normally occurs by limited proteolysis, and coagulation and complement are probably the best-characterized serine proteinase cascades in humans. Phylogenetic studies have shown that the two systems have developed more than 400 million years ago[2,3] and it has been proposed that they have evolved from a common ancestral origin in eukaryotes[4]. Notably, coagulation and complement cascades share a remarkable degree of convergent evolution with other serine protease cascades, for instance those regulating dorsal-ventral polarity in *Drosophila* (leading to an activation of Spätzle, the ligand of the Toll receptor) and the hemolymph clotting system in the horseshoe crab[4,5]. These findings suggest that the basic motifs of some proteolytic cascades existed long before the divergence of protostomes and deuterostomes[6]. It should be noted that the latter two systems (activation of Spätzle and the horseshoe crab hemolymph clotting system) are key components in ancestral immunity, which relies largely on the innate immune system. While the complement system has been considered to be part of the innate immune system for more than 30 years, it has only recently been appreciated that coagulation also partakes in inflammation and the early immune defense[7,8]. In the latter studies, a major focus has been on the ability of the clotting cascade to trigger pro- and anti-inflammatory reactions, such as the release of cytokines and activation of protease-activated receptors (PARs). However, little is known as to what extent coagulation can actively contribute to elimination of an invading microorganism.

The present study was undertaken to investigate whether activation of the coagulation system in response to bacterial infection contributes to the innate immune system and

to elimination of the invading pathogen. Special focus was placed on the role of fXIII, of which the insect homologue (transglutaminase) had recently been found to play a protective role in the immune response against bacterial pathogens in a *Drosophila* infection model[9]. *Streptococcus pyogenes* was employed in the present study, as this bacterium is considered to be one of the most important human bacterial pathogens, responsible for at least 18 million cases of severe infection worldwide (1.78 million new cases each year) and more than 500.000 deaths yearly as estimated by the WHO[10]. Infections with *S. pyogenes* are normally superficial and self-limiting, but they can develop into serious and life-threatening conditions such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS), which are associated with high morbidity and mortality[11]. The fact that *S. pyogenes* can cause local and systemic infections in the same infection model made it an ideal pathogen to be studied in the present investigation.

Methods

Bacterial strains and culture conditions

The *S. pyogenes* strains AP1 (40/58) and KTL3 of serotype M1 have been described before[12,13]. Bacteria were grown overnight in Todd-Hewitt broth (THB; Gibco, Grand Island, NY) at 37°C and 5% CO_2.

Human Plasma

Plasma obtained from healthy donors was purchased from Lund University Hospital (Lund, Sweden), plasma kallikrein- (PK)-, thrombin-, fXII, and fXIII-deficient plasma were purchased from George King Bio-Medicals Inc (Overland Park, KS).

Measurement of coagulation parameters

Activation of the intrinsic and extrinsic pathway of coagulation was determined by measuring activated partial thromboplastin time (aPTT) and prothrombin time (PT) in human or murine plasma using a coagulometer (Amelung, Lemgo, Germany) as described earlier[12].

Substrate Assays

Plasma kallikrein activity on the bacterial surface after exposure to normal, PK-, or fXIII-deficient plasma was measured using the chromogenic substrate S-2302 (Chromogenix, Milan, Italy) as previously described[12]. To measure thrombin-activity, normal, thrombin-, fXII-, or fXIII-deficient plasma were incubated with $2x10^{10}$ CFU *S. pyogenes* in 50 mM Tris supplemented with 50 µM $ZnCl_2$, 2 mM $CaCl_2$, and 1 µM phospholipids (Rossix, Mölndal, Sweden) for 30 minutes at 37°C. The tetrapeptide Gly-Pro-Arg-Pro (Bachem, Bubendorf, Switzerland) was added to prevent clotting

(1.5 mg/ml final concentration). Samples were washed with Tris and resuspended in Tris/$ZnCl_2$ and 2 mM of the chromogenic substrate S-2238. After incubation at 37°C and centrifugation, the absorbance of the supernatants was determined at 405 nm. For fXIII-activity measurement bacteria were added to normal, thrombin-, fXII-, or fXIII-deficient plasma (diluted 1/100 in sodium citrate), supplemented with zinc, calcium, and phospholipids. After incubation for 15 min at 37°C in the presence of a gold-labeled N-epsilon-gamma-glutamyl-lysine antibody [153-81D4] (GeneTex, Irvine, CA) samples were subjected to negative staining electron microscopy.

Bacterial growth in human plasma

Normal and fXIII-deficient plasma were diluted 1/100 in 12.9 mM sodium citrate and mixed with 2,5 x 10^5 CFU of *S. pyogenes.* 0.5 U thrombin from human plasma (Sigma, St. Louis, MO) were added before incubation at 37°C. After indicated time points 50 µl of the mixture were plated onto blood agar in 10-fold serial dilutions and the number of bacteria was determined by counting colonies after 18 hours of incubation at 37°C or analyzed by negative staining electron microscopy.

Generation of plasma clots

Clots from human or murine plasma (normal and fXIII-deficient) for electron microscopic analysis were prepared as previously described[9].

Crosslinking and immobilization of bacteria within the clot

Human fibrinogen (ICN Biomedicals, Aurora, OH) was incubated with M1 protein in the absence or presence of thrombin-activated human fXIII (Enzyme Research Laboratories, South Band, IN) for 30 min at 37°C. For visualization by electron

microscopy the gold-labeled N-epsilon-gamma-glutamyl-lysine antibody was added to the reaction mixture.

To analyze bacterial immobilization, clots were generated from normal and fXIII-deficient plasma, washed with PBS and covered with THB-medium. After indicated time points the 50 µl of the supernatant were plated onto blood agar as decribed above.

Electron microscopy

Samples for scanning electron microscopy were processed as described earlier[14]. Transmission electron microscopy and immunostaining using the gold-labeled N-epsilon-gamma-glutamyl-lysine antibody were performed as previously described[15]. For negative staining electron microscopy, samples were adsorbed to 400 mesh carbon-coated copper grids for 1 minute, washed with two drops of water, and stained with two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Samples were observed in a Jeol 1200 EX transmission electron microscope (Jeol, Tokyo, Japan) operated at 60kV accelerating voltage.

Animal infection model

CBA/CaOlaHsd wildtype and $fXIII^{-/-}$ mice were from Harlan (Venray, The Netherlands) and CSL Behring (Marburg, Germany), respectively. All animal experiments were approved by the regional ethical committee for animal experimentation, the Malmö/Lund djurforsöksetiska nämnd, Lund District Court, Lund, Sweden (permit M220/08). Mice were subcutaneously infected with 2.5×10^8 CFU *S. pyogenes* KTL3 as previously described[16]. After 24 hours of infection mice

were sacrificed. Skin samples were collected from the local focus of infection and fixed in 3.7% formaldehyde. For plasma analysis, citrated blood was taken from the heart at the time of sacrifice, centrifuged at 5000 rpm for 10 min, and frozen at -80°C until use. To determine bacterial loads blood and homogenates from liver and spleen were plated as described above. In some experiments mice were treated with 200 U/kg body weight of a human fXIII concentrate (Fibrogammin®P, CSL Behring) subcutaneously at the site of infection 3h after bacterial inoculation.

Examination of murine skin samples

Fixed tissue samples were dehydrated in ethanol, embedded in paraffin, and then cut into 3 µm thick sections. After de-paraffination samples were prepared for scanning electron microscopy or stained with haematoxilin and eosin (Histolab, Gothenburg, Sweden) or Giemsa (Merck, Darmstadt, Germany) for histological analysis using an Eclipse 80i microscope (Nikon, Tokyo, Japan).

Examination of human tissue biopsies

Snap-frozen tissue biopsies collected from the epicenter of infection in two patients with necrotizing fasciitis caused by *S. pyogenes* of M1T1 serotype were stained and compared with biopsies taken from healthy volunteers. The Human Subjects Review Committees of the University of Toronto and Karolinska University Hospital approved the studies, and informed consent was obtained from the patients and the volunteers. The biopsies were prepared and immunofluorescent stainings of serial sections were conducted as previously described[17,18]. The following antibodies were used in dilutions ranging from 1:250-1:10000: anti N-epsilon-gamma-glutamyl-lysine, anti-factor XIIIa (Acris, Herford, Germany), a polyclonal rabbit antiserum specific for

the Lancefield group A carbohydrate (Difco, Detroit, MI), and polyclonal rabbit antiserum against M1 protein. The immunohistochemical stainings were conducted in a RXM Leica microscope with a 25 ×/0.55 NA oil objective lens and immunofluorescent stainings in a Leica confocal scanner TCS SP II coupled to a Leica DMR microscope (Leica, Wetzlar, Germany).

Statistical analysis

Data were analyzed by using GraphPad Prism 5 (GraphPad Software, San Diego, CA). The significance between the values of an experimental group was determined by use of a variance analysis (t test).

Results

Contact activation at the surface of *S. pyogenes* leads to an induction of fXIII

Previous work has shown that the presence of *S. pyogenes* in plasma leads to an assembly and activation of the contact system at the bacterial surface[14]. It should be noted that these experiments were performed in the absence of calcium and phospholipids, which are two indispensable clotting co-factors required for an activation of coagulation factors up-stream of the contact system[19]. We therefore wondered whether calcium and phospholipid reconstitution triggers an induction of the remaining clotting cascade at the streptococcal surface. To confirm the previously reported findings we first measured the plasma kallikrein activity on AP1 bacteria upon incubation with normal human plasma supplemented with zinc. As depicted in Supplemental Figure S1A, substrate hydrolysis was monitored when bacteria were incubated with normal and fXIII-deficient plasma, but not when plasma lacked plasma kallikrein. We then set up experiments to monitor whether bacteria-induced contact activation leads to an induction of the entire coagulation cascade by measuring thrombin activity, the activator of fXIII. To this end, normal plasma was reconstituted with zinc, calcium, and phospholipids. Samples were also supplemented with a tetrapeptide (Gly-Pro-Arg-Pro) to avoid polymerization of thrombin-generated fibrin monomers and subsequent clot formation (for detailed information see *Methods*). When this reaction mixture was added to normal plasma and incubated with AP1 bacteria, an increase of thrombin activity at the bacterial surface was monitored (Supplemental Figure S1B). Similar results were also obtained with fXIII-deficient, but not with fXII-deficient or thrombin-deficient plasma (Supplemental Figure S1B), implying that activation of the contact system at the bacterial surface is required to trigger activation of the remaining clotting factors.

fXIII is one of thrombin's substrates and we therefore tested whether thrombin activation induced by bacteria triggers a conversion of fXIII into its active form. To this end, we employed immunoelectron microcopy with an antibody directed against N-epsilon-gamma-glutamyl-lysine which specifically recognizes amino acids that are covalently crosslinked by the action of fXIII[20]. Since Gly-Pro-Arg-Pro exerts a mild bacteriostatic effect in our experiments, we decided not to use this peptide as an anti-coagulant. Instead, plasma was diluted (1/100) to generate a fibrin concentration which is too low to cause its polymerization when activated by thrombin. Bacteria were incubated with diluted normal, thrombin-, fXII-, and fXIII-deficient plasma in the presence of the gold-labeled antibody, zinc, calcium, and phospholipids. Samples were subsequently analyzed by negative staining electron microscopy. Figure 1A shows antibody binding to the surface of *S. pyogenes* bacteria treated with normal diluted plasma, while only background signals were detected when bacteria were incubated with fXII- or fXIII-deficient plasma (Fig. 1A). Similar results were obtained with thrombin-deficient plasma (data not shown). Taken together these results suggest that contact activation at the bacterial surface can evoke an induction of the entire coagulation cascade and eventually enables fXIII to act on *S. pyogenes* surface proteins.

Streptococci are killed in thrombin-activated plasma

It has been shown that contact activation on the surface of *S. pyogenes* leads to the generation of antimicrobial peptides[21]. Therefore, we wished to study the fate of crosslinked bacteria in activated, but non-clotted, normal and fXIII-deficient plasma. Our results show that bacterial growth is significantly impaired in thrombin-activated normal and fXIII-deficient plasma (diluted 1/100). This effect was time-dependent

and dependent on plasma activation (Figure 1B). To study whether these results are due to an induction of antimicrobial activity, plasma-treated bacteria were subjected to negative staining electron microscopy. Supplemental Figure S1C depicts intact bacteria that were incubated with non-activated normal plasma, and similar findings were observed when bacteria were incubated with non-activated fXIII-deficient plasma (Supplemental Figure S1C). In the presence of thrombin, however, incubation with normal or fXIII-deficient plasma caused multiple disruptions of the bacterial cell wall and triggered an efflux of cytosolic content (Supplemental Figure S1C) which is a sign of bacterial killing[17]. Notably, incubation of *S. pyogenes* with thrombin in the absence of plasma neither impaired bacterial growth (Figure 1B) nor did it cause cytosolic leakage (data not shown).

To test whether bacterial killing also occurs within a formed clot, AP1 bacteria and undiluted plasma were mixed and thrombin was added. The clots formed were incubated for 1 h, thin-sectioned and analyzed by transmission electron microscopy. Figure 1C displays that a significant number of bacteria in clots generated from normal and fXIII-deficient plasma are devoid of cytosolic content, suggesting a substantial disruption of the cell membrane and bacterial killing. By contrast only a few dead bacteria were seen when clots were thin-sectioned directly after the addition of thrombin. Statistical analysis revealed an efficient killing of bacteria within the clot regardless of whether the clot was formed from normal or fXIII deficient plasma (6% at time 0 h and 35% after 1 h for normal plasma and 5% at time 0 h and 36% after 1 h for fXIII deficient plasma). Together these data demonstrate that activation of the coagulation cascade on the surface of *S. pyogenes* leads to a fXIII independent induction of antimicrobial activity.

Bacterial entrapment within a plasma clot is fXIII-dependent

It was recently shown that human fXIII crosslinks and immobilizes bacteria of the species *Staphylococcus aureus* and *Escherichia coli* inside a plasma clot[9]. To test whether this also applies to *S. pyogenes*, bacteria of strain AP1 were incubated with normal and fXIII-deficient plasma. After activation with thrombin, clots were analyzed by scanning electron microscopy. Figures 2A and 2B show clots formed from normal and fXIII-deficient plasma in the absence of bacteria. The micrographs reveal that both types of clots share a similar morphology, although clots generated from fXIII-deficient plasma appear less dense. However, dramatic changes were observed when clots were formed in the presence of *S. pyogenes* AP1 bacteria. While massive loads of bacteria were entrapped in clots derived from normal plasma (Figure 2C), only a few bacteria were found attached to clots when fXIII-deficient plasma was used (Figure 2D). Also, fibrin network formation was reduced when bacteria were incubated with normal plasma, which was not seen in fXIII-deficient plasma (Figure 2C and 2D). At higher resolution it is noticeable that fibrin fibers and bacteria are in close proximity in the clots generated from normal plasma and it even appears that fibers originate from the bacterial surface (Figure 2E). By contrast, bacteria are loosely assembled in clots from fXIII-deficient plasma and no direct interaction with fibrin fibers is visible (Figure 2F). To confirm these findings, clots from normal plasma were thin-sectioned and subjected to transmission electron microscopy, which allows an analysis at higher resolution. Figure 2G-I depicts thin-sectioned *S. pyogenes* AP1 bacteria before (Figure 2G) and directly after incubation with normal plasma and subsequent thrombin-activation (Figure 2H). Within the clot, bacteria are strung along fibrin fibers and it appears that they have multiple interaction sites. Additional immunostaining with the gold-labeled antibody against N-epsilon-gamma-glutamyl-

lysine was used to study the mode of interaction between bacteria and fibrin fibers. As expected, numerous crosslinking events within fibrin fibers were detected. The electron microscopic analysis also revealed that fibrin fibers are avidly crosslinked to the bacterial surface (Figure 2I). Crosslinking activity was not recorded when bacteria were incubated with fXIII-deficient plasma (data not shown).

Most streptococcal serotypes have a high affinity for fibrinogen and the M1 protein has been reported to be the most important fibrinogen receptor of the *S. pyogenes* AP1 strain[22]. The respective binding sites were mapped to the amino-terminal region of M1 protein and fragment D, which is part of the terminal globular domain of fibrinogen[22]. Negative staining electron microscopy was employed to study the interaction between M1 protein and fibrinogen at the molecular level. The results demonstrate that one terminal region of the streptococcal surface protein is in complex with a globular domain of fibrinogen (Figure 3A, *upper panel*), which is in good agreement with the mapping study. The nature of this complex was not altered when activated fXIII was co-incubated with the two proteins (Figure 3A, *middle panel*). Indeed, additional immuno-detection with the gold-labeled antibody against N-epsilon-gamma-glutamyl-lysine revealed that the interaction site is covalently crosslinked by fXIII (Figure 3A, *lower panel*). M proteins are the most abundant surface proteins of streptococci and it is therefore plausible that M1 protein of *S. pyogenes* AP1 bacteria is one of the major interaction partners that is covalently attached to fibrin fibers by the action of fXIII. However, it cannot be excluded that other streptococcal surface proteins are also targeted by fXIII.

Whether crosslinking of bacteria by fXIII has a pathophysiologic function inside the clot, was studied by measuring the escape of *S. pyogenes* AP1 bacteria from clots generated from normal and fXIII-deficient plasma. To this end streptococci were

mixed with undiluted normal or fXIII-deficient plasma and clotting was induced by the addition of thrombin. Clots were then washed with PBS and covered with growth medium. After different time points samples were collected from the supernatant and the bacterial load was determined. As seen in Figure 3B, fXIII-induced crosslinking significantly reduced the release of bacteria from the clot suggesting that they are immobilized and killed within the clot. Taken together, the results show that *S. pyogenes* bacteria are covalently woven into a fibrin network by the action of fXIII and their dissemination from the clot is prevented.

S. pyogenes infected fXIII$^{-/-}$ mice show more signs of inflammation than wildtype animals at the local focus of infection

The *in vitro* data suggest that coagulation is part of the early innate immune response, which in a concerted action triggers the immobilization and killing of *S. pyogenes* inside a clot. We therefore hypothesized that prevention of bacterial dissemination and their clearance may dampen the inflammatory response at the site of infection. To test this, we took advantage of a skin infection model that was established with another *S. pyogenes* strain of M1 serotype, namely KTL3[16]. Challenge with *S. pyogenes* KTL3 normally causes local infections that eventually disseminate from the infection focus and lead to systemic infection[16]. By employing scanning electron microscopic analysis, we found that incubation of *S. pyogenes* KTL3 with thrombin-activated human normal or fXIII-deficient plasma *in vitro*, generates clots with a morphology similar to those generated with *S. pyogenes* AP1 bacteria (data not shown). Similar results were also obtained when murine plasma (normal and fXIII-deficient) was incubated with *S. pyogenes* KTL3 bacteria (Supplemental Figure S2).

To study the inflammatory response to local infection with *S. pyogenes*, wildtype and $fXIII^{-/-}$ mice were subcutaneously infected with *S. pyogenes* KTL3. 24 h after infection, mice were sacrificed and the skin from the local focus of infection was surgically removed and stained for histopathological analysis. Microscopic examination of hematoxilin/eosin stained skin biopsies from non-infected wildtype and $fXIII^{-/-}$ mice revealed no signs of inflammation (Figure 4A and 4B), while cell invasion, and tissue damage were seen in biopsies from infected wildtype animals (Figure 4C). Notably, these lesions were far more severe in biopsies from infected $fXIII^{-/-}$ mice (Figure 4D). Biopsies were also stained with Giemsa to detect infiltrating cells. Analysis of tissue samples from wildtype animals shows that bacteria were found in clustered patches and some neutrophils have been recruited (Fig. 4E). In biopsies from $fXIII^{-/-}$ mice, bacteria were scattered throughout the whole infection site and an increased number of neutrophils was detected (Fig. 4F).

Further electron microscopy examination of the tissue biopsies revealed severe bleeding across the infected site in both wildtype and $fXIII^{-/-}$ mice (data not shown). Bacteria were found entrapped and clustered within the fibrin meshwork of infected wildtype mice (Figure 4E), whereas bacteria were distributed throughout the whole infection site in skin biopsies from infected $fXIII^{-/-}$ mice (Figure 4F). Additional statistical analysis revealed approximately 8 bacterial clusters per 100 μm^2 in the fibrin network of wildtype animals (Figure 4G), while streptococci were mostly seen as single bacteria or small chains at a density of 41 bacteria/chains per 100 μm^2 in $fXIII^{-/-}$ animals (Figure 4H). At higher magnification it appears that bacteria are an integral part of the fibrin network from infected wildtype mice (Figure 4G, *insert*). This was not observed in biopsies from $fXIII^{-/-}$ mice where streptococci were found associated with, but not as a constituent part of the network (Figure 4H, *insert*). In

order to assess the contribution of immobilization of bacteria to their dissemination, clotting times of the intrinsic pathway of coagulation (activated partial thromboplastin time or aPTT) were measured. Increased aPTTs are a sign of a systemic response to the infection[12]. Plasma samples were recovered 24h after infection and clotting times of the intrinsic pathway of coagulation were determined. Figure 4I shows that the aPTTs of plasma samples from infected wildtype mice were moderately but significantly increased, while clotting times were extremely high in plasma samples from $fXIII^{-/-}$ mice. The prothrombin time (PT) remained unaltered after 24 h of infection in both groups of mice (data not shown). Together these results demonstrate that the lack of fXIII leads to an increased inflammatory response at the infectious site combined with an induction of systemic reactions.

fXIII crosslinking in patients with necrotizing fasciitis caused by *S. pyogenes*

To test whether the results obtained from the animal studies also apply to the clinical situation, biopsies from patients with necrotizing fasciitis caused by *S. pyogenes* were analyzed by immunohistology and electron microscopy. Figure 5 depicts massive tissue necrosis at the site of infection and subsequent immunodetection in serial tissue sections showed positive staining for the M1 protein and fXIII at these sites. This suggests an influx of plasma to the infected focus and indeed crosslinking activity at the same location was recorded (Figure 5, *upper lane*). As controls, biopsies from healthy persons were used, in which no signal was seen when subjected to the same experimental protocol (Figure 5, *lower lane*). Tissue sections were further analyzed by confocal immuno-fluorescence microscopy using antibodies against M1 protein and N-epsilon-gamma-glutamyl-lysine. Figure 6A shows positive staining for M1 protein and wide-spread positive staining for N-epsilon-gamma-glutamyl-lysine. In

addition we found a striking co-localization of the two antibodies (Figure 6A), suggesting bacterial crosslinking at the infected site. When the biopsies were analyzed by scanning electron microscopy, massive bleeding at the infected site was recorded (data not shown) and bacteria were found clustered and entrapped inside the fibrin network (Figure 6B). Specimens were also thin-sectioned and studied by immuno transmission electron microscopy using the gold-labeled antibody against N-epsilon-gamma-glutamyl-lysine. Figure 6C shows immuno-staining at the bacterial surface in regions that are in contact with fibrin fibers. The micrographs also reveal that a significant portion (31%) of the entrapped bacteria were not viable as shown in figure 6D. These findings are in line with the *in vitro* and *in vivo* experiments and they illustrate that immobilization of bacteria and generation of antimicrobial activity is seen within the fibrin network in patients with severe and invasive infections with *S. pyogenes*.

Local treatment with fXIII dampens systemic bacterial spreading in infected mice

To test whether treatment with fXIII is able to prevent bacterial spreading in an animal model of infection, wildtype mice were subcutaneously infected with *S. pyogenes*. Three hours after challenge, half of the mice were treated with Fibrogammin®P, a human plasma fXIII concentrate, which was injected into the site of infection. A dose of 200 U per kg body weight was chosen, which is well tolerated in mice and gives rise to approximately 250% of total fXIII activity when injected intravenously[23]. Mice infected with *S. pyogenes* but without Fibrogammin®P-treatment served as controls. Infected animals were sacrificed after 24h of infection and bacterial loads in blood, liver, and spleen were determined. As depicted in Figure

7, Fibrogammin®P-treatment resulted in decreased bacterial loads in blood, liver, and spleen of the treated mice, suggesting fXIII dampens systemic dissemination of *S. pyogenes* in the infected animals. Taken together, the results presented in this study support the concept that fXIII has an important role in the early immune response to bacterial infections. We show that fXIII triggers an immobilization of bacteria within the fibrin network at the local focus of infection, which is combined with an induction of plasma-derived antimicrobial activity and subsequent bacterial killing. The two mechanisms work in concert and may together diminish early bacterial dissemination and down-regulate the inflammatory response.

Discussion

Sensing the first signs of inflammation and rapid elimination of an invading microorganism are key features of the early immune response to infection. In particular, potential ports of microbial entry are at great risk and therefore need special protection. Thus, the immune system has developed mechanisms that allow an efficient clearance of inhaled (for example with the help of mannose-binding lectin[24]) or swallowed (for example by the action of intestinal mucins[25]) pathogens. Wounds present another port of entry and are associated with a great risk to promote infections and allow microorganisms to enter the circulatory system. To prevent their dissemination and eventual systemic complications, it is of great importance that the host's defense system is activated as soon as wound sealing begins. It therefore appears likely that coagulation plays an important role in these very early processes. However, the extent and underlying mechanisms of this contribution to immunity are poorly understood.

Here we show for the first time that, in addition to its proinflammatory role, coagulation plays an active role in the containment and elimination of bacteria in infections caused by *S. pyogenes*. Our data support a model based on two separate mechanisms, involving a fXIII-triggered covalent immobilization of microorganisms inside the fibrin network and the generation of antimicrobial activity. We find that clotting is activated at the bacterial surface via the intrinsic pathway of coagulation also referred to as the contact system or kallikrein/kinin system. Apart from bacteria[26], also fungi[27] and viruses[28] have been reported to interact with the contact system, supporting the notion that contact activation is subject to the principles of pattern recognition[29]. Notably, the system is activated within seconds and leads to the release of antimicrobial peptides[21,30] and inflammatory mediators[31] further supporting its role

in early innate immunity. In addition to generation of antimicrobial peptides due to activation of the intrinsic pathway of coagulation, processing of thrombin has recently been shown to release host defense peptides with a broad specificity[32]. However, the extent to which theses peptides contribute to the antimicrobial activity seen in the present study and the ability of these peptides to kill other bacterial species needs to be clarified.

The *in vivo* data presented in this investigation, show that the lack of fXIII evokes pathologic inflammatory reactions illustrated by a massive neutrophil influx to the site of infection and subsequent tissue destruction as seen in the infected mice. The inability to immobilize bacteria in a fibrin network, leads to a dramatic increase of the intrinsic-driven clotting time in these animals, which is an indication of more severe systemic infection in the knock-out when compared to wildtype mice. These findings are in line with a recent report by Sun *et al.*[33]. The authors used mice deficient in factor V or fibrinogen to show that local thrombosis/fibrin deposition limits the survival and dissemination of group A streptococci[33]. Notably, many bacterial pathogens are able to activate plasminogen at their surface by employing different modes of action[34]. This mechanism would allow bacteria to escape their entrapment in a fibrin network. Indeed it has been reported for instance for *Yersinia pestis* that mutant strains lacking the plasminogen activator Pla, failed to cause an otherwise systemic infection when tested in an subcutaneous murine infection model[35].

Human plasma fXIII is fully active in mice[23] and as a proof of concept we administered the human protein in a murine infection model. When wildtype mice were treated with human plasma fXIII, dissemination of *S. pyogenes* was significantly reduced when compared to non-treated mice. Recent findings showing that fXIII also crosslinks surface proteins from other bacterial species, such as *Escherichia coli* and

Staphylococcus aureus bacteria[9], imply that the mechanism described herein is an important part of the early immune response. Our results underline the importance of fXIII in the early defense against *S. pyogenes* and they suggest that fXIII is an interesting target for the development of novel antimicrobial therapies.

Clotting has been previously implicated in immunity in invertebrate models, where its immune function is more visible due to the lack of redundancy with adaptive effector mechanisms. One of the best studied examples is the clotting system of horseshoe crabs, which is triggered by minute amounts of bacterial elicitors, such as LPS. This leads to the production of antimicrobial activity and communicates with other effector systems. In a similar way there may be cross-talk between complement and blood clotting for example via the binding of ficolin to fibrin/fibrinogen[36]. The picture that emerges from evolutionary comparisons is that proteolytic cascades and their constituent proteases are used as flexible modules, which can be triggered by endogenous as well as exogenous microbial elicitors[37]. Even one and the same proteolytic event can be activated by distinct elicitors in different contexts. One such example is the cleavage of the *Drosophila* protein Spätzle, which can act as a key signal both during development and in the immune system. In both cases cleaved Spätzle binds to Toll, the founding member of the TLR family. In a similar way we show here that blood clotting, which has so far been mostly been studied in the context of its physiological hemostatic function, plays a key role in immunity both as an effector mechanism and by communicating with other branches of the immune system. This leads to a fast and efficient instant immune protection, which keeps infections localized and leaves additional time for other effector mechanisms to be activated[5].

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Authorship

Contribution: T.G.L. performed research, analyzed the data, and wrote the paper; M.M. contributed analytic tools and performed research; L.J., S.O., and A.I.O. performed research; G.D. and A. N.-T. contributed analytic tools; U.T. and H.H. designed research and wrote the paper.

Conflict-of-interest disclosure: CSL Behring GmbH (Marburg, Germany) and Hansa Medical AB (Lund, Sweden) are in the process of filing a patent application on fXIII. GD, HH, MM, TGL, and UT are listed as inventors. The remaining authors declare no competing financial interests.

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Figure Legends

Figure 1: The contact system is activated on the bacterial surface after exposure to plasma leading to antimicrobial activity

(A) *S. pyogenes* AP1 bacteria were incubated in sodium citrate alone, normal plasma, fXII-, or fXIII-deficient plasma (all diluted 1/100 in sodium citrate) in the presence of $ZnCl_2$, $CaCl_2$, phospholipids, and the gold-labeled antibody against N-epsilon-gamma-glutamyl-lysine for 15 min and afterwards analyzed by negative staining electron microscopy. The scale bar represents 100 nm.

(B) *S. pyogenes* AP1 bacteria were incubated in non-activated or thrombin-activated normal and fXIII-deficient plasma (1/100 diluted). After indicated time points bacterial numbers were determined by plating of serial dilutions onto blood agar. Bacteria incubated in sodium citrate in the presence of thrombin served as controls. The figure represents the mean ± SD of three independent experiments. ** $P < 0.01$; *** $P < 0.001$.

(C) AP1 bacteria were incubated with normal or fXIII-deficient plasma and clotting was initiated by the addition of thrombin. Thin sectioned clots before (0h) and after 1 h of incubation at 37°C are shown. Similar amounts of dead bacteria (arrows) were detected in both samples after incubation. The scale bar indicates 1 µm.

Figure 2: Entrapment and immobilization of *S. pyogenes* inside the clot

Scanning electron micrographs showing the structure of clots generated from normal plasma (A, C, E) or fXIII-deficient plasma (B, D, F) in the absence (A, B) or presence (C – F) of bacteria. The scale bars represent 10 µm in A-D and 1 µm in E-F, respectively. The transmission electron micrographs depict *S. pyogenes* alone (G), after exposure to thrombin-activated plasma (H), and after exposure to plasma

followed by immunostaining with a gold-labeled N-epsilon-gamma-glutamyl-lysine antibody recognizing the fXIII crosslinking site (I). Scale bars correspond to 1 µm in G and H, and to 100 nm in I, respectively.

Figure 3: fXIII crosslinks the streptococcal M1 protein with fibrinogen leading to immobilization of bacteria within the clot

(A) The electron micrographs show negatively stained human fibrinogen (characterized by three domains) in complex with M1-protein (elongated) before (*upper panel*) and after fXIII crosslinking (*middle panel*). Crosslinking was detected by immunostaining the fibrinogen M1 protein complex with the gold-labeled antibody against N-epsilon-gamma-glutamyl-lysine (*lower panel*). A schematic drawing of the fibrinogen (grey) and M1 protein (black) is included to highlight the interaction between fibrinogen and M1 protein. The scale bars represent 25 nm.

(B) Bacteria were incubated with normal or fXIII-deficient plasma and clotting was initiated by the addition of thrombin. Clots were washed briefly, covered with THB-medium and further incubated at 37°C. After indicated time points bacterial numbers were determined by plating of serial dilutions of the supernatant onto blood agar. The figure represents the mean ± SD of three independent experiments. * $P < 0.05$; *** $P < 0.001$.

Figure 4: Subcutaneous infection of wildtype and fXIII$^{-/-}$ mice with *S. pyogenes*

Haematoxilin/eosin stained representative tissue sections from non-infected (A, B) and infected (24 h; C, D) wildtype (A, C) and fXIII$^{-/-}$ (B, D) mice are shown. Infected animals show signs of inflammation (white arrows) and tissue damage (black arrows). The scale bar represents 500 µm.

Giemsa stained biopsies from the inflamed area of wildtype (E) and fXIII$^{-/-}$ mice (F) are shown. Arrowheads point to infiltrating inflammatory cells (macrophages/neutrophils). The scale bar represents 25 µm.

Scanning electron micrographs depict biopsies from wildtype (G) and fXIII$^{-/-}$ (H) mice. Arrows indicate bacteria entrapped and clustered within the fibrin meshwork in wildtype mice (G), and scattered throughout the infection area in fXIII$^{-/-}$ animals (H). Scale bars correspond to 10 µm and to 1 µm in the inserts.

(I) Activated partial thromboplastin time (aPTT) measured in plasma from non-infected and infected wildtype and fXIII$^{-/-}$ mice (24 h after infection). Data are presented as mean value of plasma samples obtained from 3 or 5 non-infected and 9 infected animals obtained from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

Figure 5: Immunohistochemical analysis of human biopsies

Tissue biopsies were obtained from patients with necrotizing fasciitis caused by *S. pyogenes* (*upper panel*) and healthy volunteers (*lower panel*). The biopsies were sectioned and immunohistochemically stained for streptococcal M1-protein, fXIII, and N-epsilon-gamma-glutamyl-lysine. Stainings without primary antibodies were negative (data not shown). The scale bars correspond to 50 µm.

Figure 6: Co-localization of M1 protein and the fXIII crosslinking site in human biopsies

(A) Tissue biopsies from patients with streptococcal necrotizing fasciitis were sectioned and immunofluorescently stained for M1 protein (green) in combination with anti N-epsilon-gamma-glutamyl-lysine (red). Confocal microscopy revealed co-

localization of both antibodies, seen at higher magnification in inset figure. Cell nuclei are stained in blue with DAPI.

(B) Scanning electron microscopy showing bacteria entrapped in the fibrin network (arrows) in a biopsy from a patient with streptococcal necrotizing fasciitis. Scale bar indicates 5 µm.

(C) Transmission electron micrograph displaying fXIII-mediated crosslinking of bacterial surface proteins to fibrin by detection of the gold-labeled antibody against N-epsilon-gamma-glutamyl-lysine. The scale bar represents 100 nm.

(D) Transmission electron microscopy shows dead bacteria inside the fibrin network in a biopsy from a patient with streptococcal necrotizing fasciitis. The scale bar represents 0.5 µm.

Figure 7: fXIII-Treatment of wildtype mice and bacterial dissemination

Mice received a subcutaneous injection of *S. pyogenes* and were treated with Fibrogammin®P 3 h after infection. Non-treated mice served as control. 24 h after infection mice were sacrificed and bacterial loads in (A) blood, (B) liver, and (C) spleen were determined. Data are presented as mean of 10 mice per group and obtained from three independent experiments. * $P < 0.05$; ** $P < 0.01$.

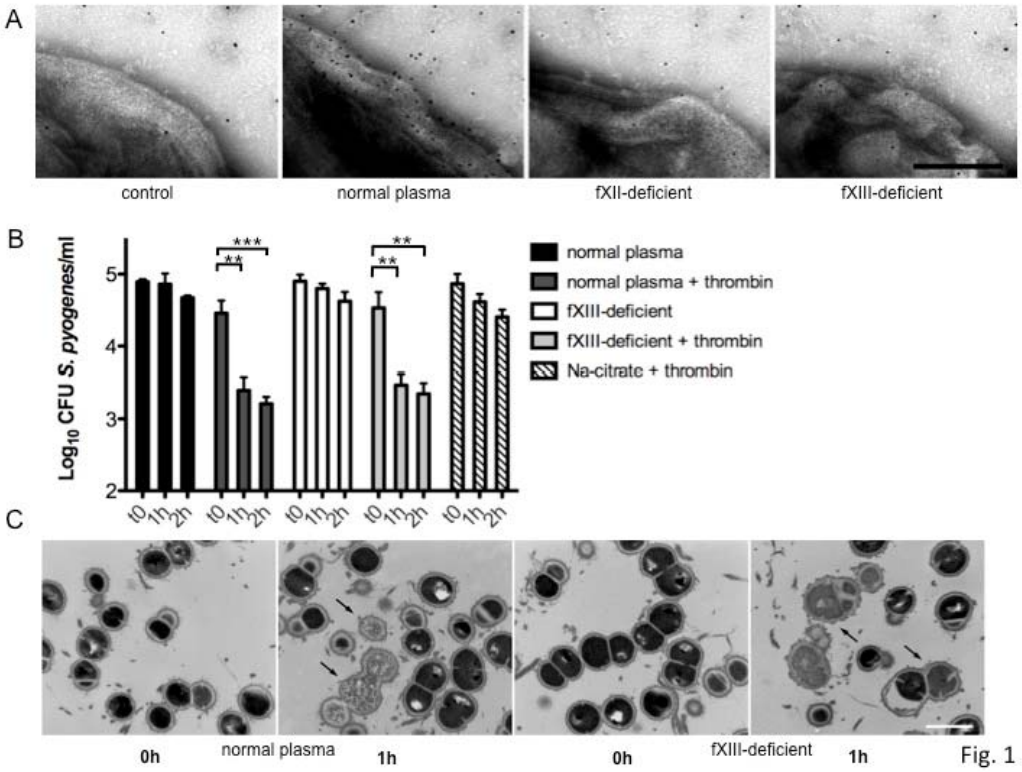

A
control
normal plasma
fXII-deficient
fXIII-deficient
B
Log10 CFU S. pyogenes/ml
5
4
3
2
***
**
**
**
t0 1h 2h t0 1h 2h t0 1h 2h t0 1h 2h
normal plasma
normal plasma + thrombin
fXIII-deficient
fXIII-deficient + thrombin
Na-citrate + thrombin
C
0h
normal plasma
1h
0h
fXIII-deficient
1h

Fig. 1

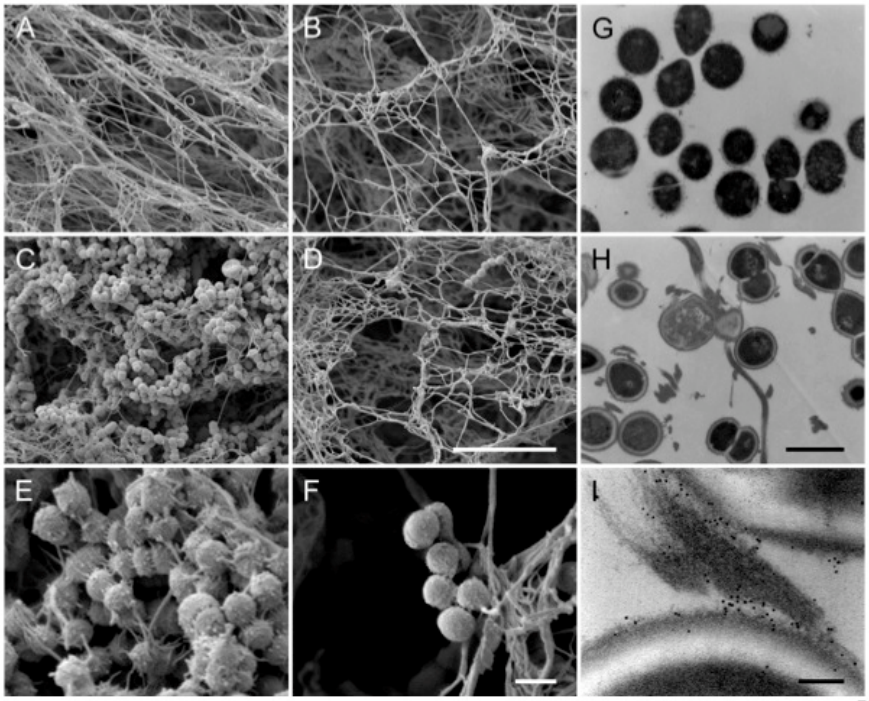


Fig. 2

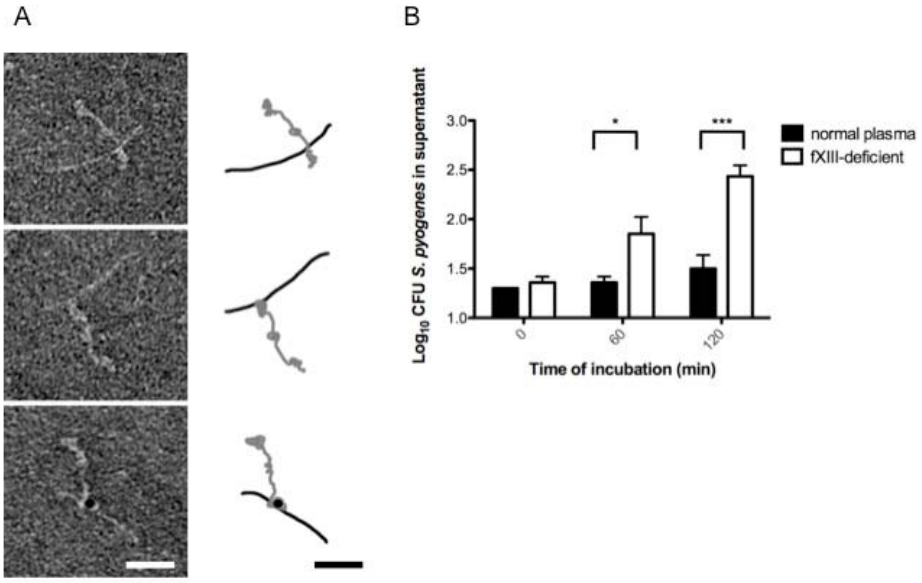

A
B
Log10 CFU S. pyogenes in supernatant
3.0
2.5
2.0
1.5
1.0
*
***
normal plasma
fXIII-deficient
0
60
120
Time of incubation (min)

Fig. 3

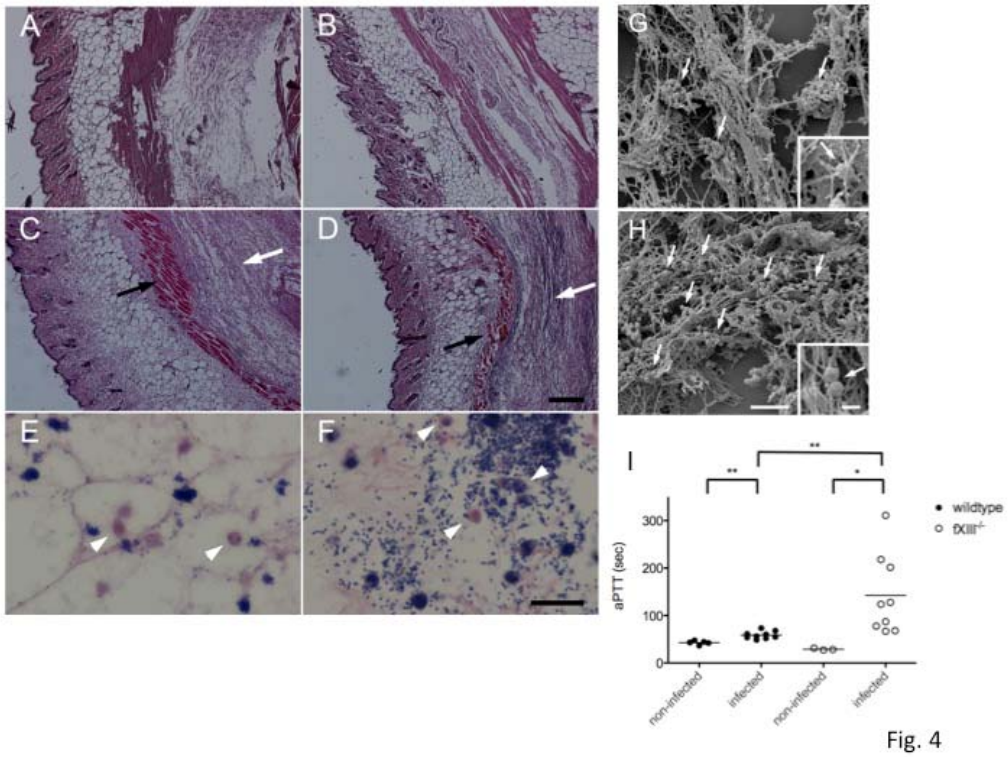

A
B
C
D
E
F
G
H
I
**
**
*
300
200
100
0
aPTT (sec)
wildtype
FXIII-/-
non-infected
infected
non-infected
infected

Fig. 4

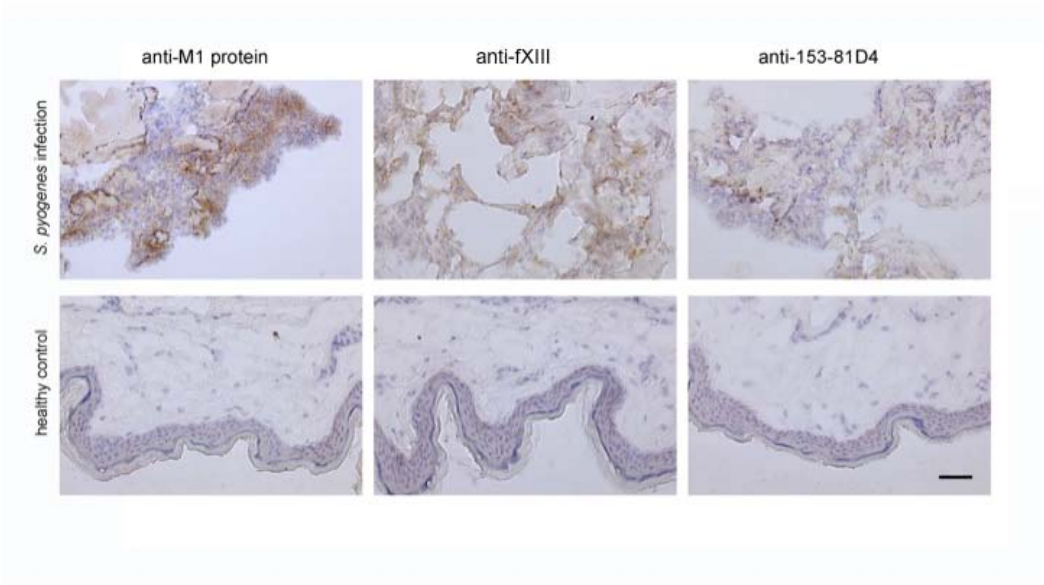

anti-M1 protein
anti-fXIII
anti-153-81D4
S. pyogenes infection
healthy control

Fig. 5

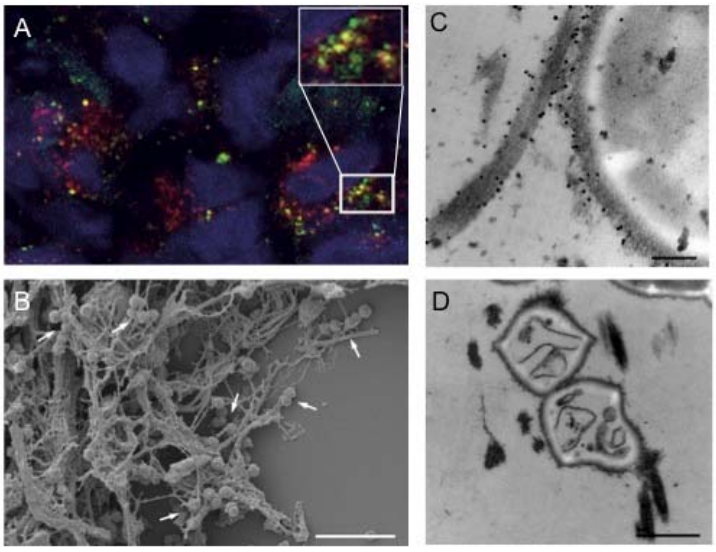


Fig. 6

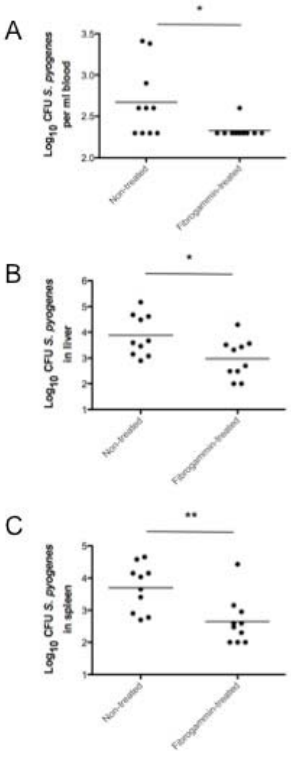
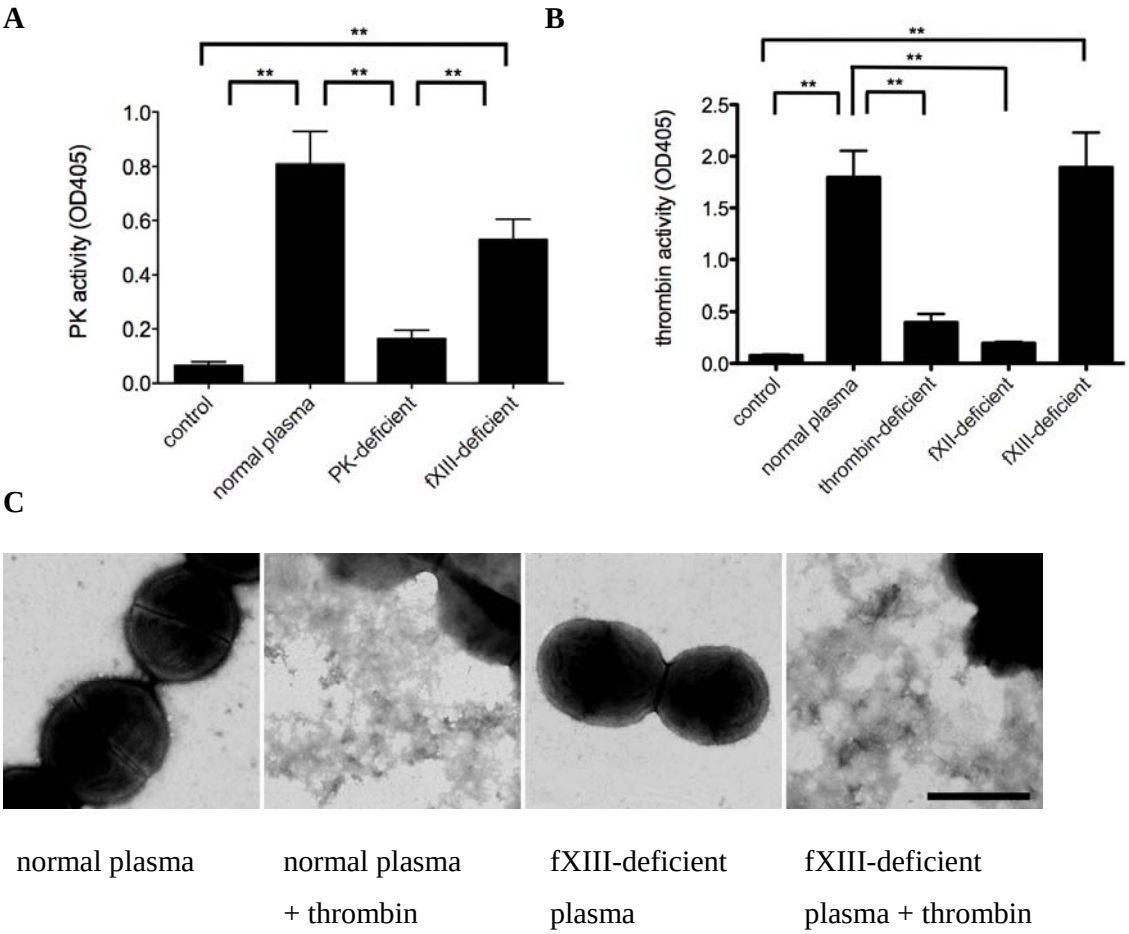

A
*
Log10 CFU S. pyogenes per ml blood
3.5
3.0
2.5
2.0
Non-treated
Fibrogammin-treated
B
*
Log10 CFU S. pyogenes in liver
6
5
4
3
2
1
Non-treated
Fibrogammin-treated
C
**
Log10 CFU S. pyogenes in spleen
5
4
3
2
1
Non-treated
Fibrogammin-treated

Fig. 7



Supplemental Figure S1: The contact system is activated on the bacterial surface after exposure to plasma

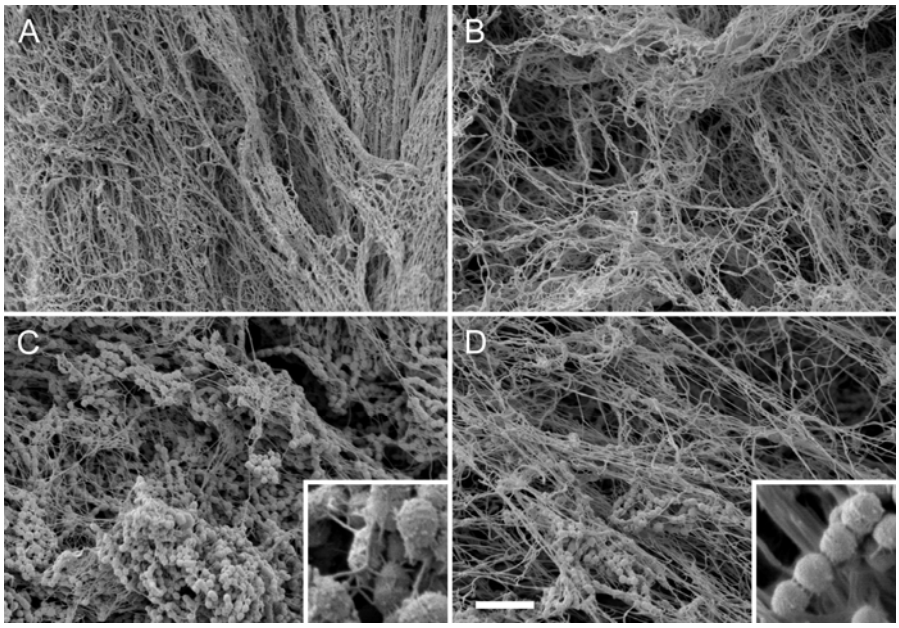
(A) Activated partial thromboplastin time (aPTT) measured in normal, PK-deficient, fXII-deficient, and fXIII-deficient plasma. Data are presented as mean ± SD value of plasma samples obtained from three independent experiments. *** $P < 0.001$.

(B) AP1 bacteria in Tris containing $ZnCl_2$ were incubated with human normal, PK-deficient, or fXIII-deficient plasma for 30 min. Bacteria were then washed and resuspended in a substrate solution for the measurement of the plasma kallikrein activity on the surface of *S. pyogenes*. The figure represents the mean ± SD of three

independent experiments. ** $P < 0.01$.

(C) *S. pyogenes* in Tris containing $ZnCl_2$ were incubated with normal, thrombin-, fXII-, and fXIII-deficient plasma in the presence of $CaCl_2$ and phospholipids for 30 min. Bacteria were washed and resuspended in a substrate solution to measure the thrombin activity. The figure represents the mean ± SD of three independent experiments. ** $P < 0.01$.

(D) AP1 bacteria were incubated in normal plasma, thrombin-activated normal plasma, F XIII-deficient plasma, or thrombin-activated F XIII-deficient plasma as described in *Methods* and subjected to analysis by negative staining electron microcopy. The scale bar represents 1 µm.



Supplemental Figure S2: F XIII-dependent entrapment of *S. pyogenes* KTL3 in clots generated from murine plasma.

Scanning electron micrograph displaying FXIII-dependent entrapment of *S. pyogenes* in clots generated from murine plasma. Plasma obtained from wildtype (A, C) and $FXIII^{-/-}$ mice (B, D) was incubated in the absence and presence of 2 x 10^9 CFU of *S. pyogenes* strain KTL3 and clotting was initiated by the addition of thrombin. Similar to the results with human plasma, large amounts of *S. pyogenes* are captured within the clot generated from wildtype (normal) plasma (C) whereas only some few bacteria are found in the FXIII-deficient clot (D). A closer view on the bacteria revealed strong interactions of the surface of *S. pyogenes* with the fibrin network in the wildtype, but not in the clot lacking FXIII (insets in C and D). The scale bar represents 10 µm respectively 1 µm in the inserts.