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**Treatment of invasive streptococcal infection with a peptide derived
from human high molecular weight kininogen**

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Abstract

Sepsis and septic shock remain an important medical problem, emphasizing the need to identify novel therapeutic opportunities. Hypovolemic hypotension, coagulation dysfunction, disturbed microcirculation, and multiorgan failure due to vascular leakage are often observed in these severe conditions. In the present study, we find that HKH20, a peptide derived from human high molecular weight kininogen (HK), down-regulates inflammatory reactions caused by *Streptococcus pyogenes* in a mouse model of sepsis. HK is a component of the pro-inflammatory and pro-coagulant contact system. Activation of the contact system in the bloodstream by *S. pyogenes* leads to massive tissue damage in the lungs of the infected mice, which eventually results in the death of the animals. HKH20 inhibits activation of the contact system and protects mice with invasive *S. pyogenes* infection from lung damage. In combination with clindamycin treatment, the peptide also significantly prolongs the survival of infected mice.

Introduction

Sepsis and septic shock are complications of bacterial infections that are - despite treatment with antibiotics and improved intensive care - associated with high mortality rates. *Streptococcus pyogenes* is a major human pathogen that mainly causes skin and throat infections. These infections are normally superficial and self-limiting, but they occasionally develop into the serious and life-threatening conditions streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis (for a review see ¹). The molecular mechanisms behind the pathogenesis of these critical conditions are still not fully understood. However, a growing body of evidence suggests that they are the result of an uncontrolled host inflammatory response induced by the pathogen. A systemic activation of proteolytic host cascades such as the complement, coagulation, and contact systems, plays an important role, together with a massive release of pro-inflammatory cytokines ².

Previous work has shown that *Streptococcus pyogenes* is able to assemble and activate the human contact system on its surface ³. The contact system, also known as the intrinsic pathway of coagulation or the kallikrein-kinin system, is involved in normal hemostasis and inflammation ⁴⁻⁶. It comprises four components: Factor XI (FXI), FXII, plasma kallikrein (PK) and high molecular weight kininogen (HK). Under physiological conditions, these factors circulate in their inactive forms in the bloodstream or are bound to the surface of different cell types, such as endothelial cells, platelets and polymorphonuclear neutrophils (PMNs). Upon activation, the contact system triggers the intrinsic pathway of coagulation via activation of Factor XI by FXII and evokes the release of bradykinin (BK) from the HK precursor by the action of PK. BK, a peptide consisting of nine amino acids, is a potent pro-inflammatory mediator. Thus, BK has been shown to evoke the generation of nitric oxide (NO) and other inflammatory substances (for instance prostaglandins and leukotrienes), reduce blood pressure, and induce fever. Notably, and probably more important in respect to infectious diseases, BK also induces increased vascular permeability and capillary leakage, causing pain, edema and hypotension ^{4,6}. BK levels are often significantly increased in patients with sepsis and septic shock. While the local activation of the contact system is considered to have a beneficial effect for the human host, i.e. via generation of HK-derived antibacterial peptides ⁷, a systemic contact activation may lead to severe complications such as kinin induced vascular leakage and bleeding disorders ⁸. Several reports have described contact system activation in various animal models of infection with different pathogens ⁹⁻¹³. Contact activation has been seen in all animal species tested including baboons and rats ^{9,11} and also in mice, where the degree of activation varies between mouse strains ¹³.

Thus, animal models of infection may be useful tools to study contact system inhibition for therapeutic purposes.

The present study investigates whether a peptide (HKH20) derived from a region of HK known to interact with bacterial surfaces, could be used to block the activation of the contact system and to treat experimental *S. pyogenes* infections. The results show that HKH20 is a potent inhibitor of the contact system. Moreover, in a mouse model of invasive *S. pyogenes* infection, the peptide prevented pulmonary lesions. In combination with clindamycin, HKH20 significantly improved the survival rate during murine infection.

Material and Methods

Materials

The *S. pyogenes* strain AP1 (40/58) of serotype M1 was from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. Bacteria were grown in Todd-Hewitt broth (TH; Difco) at 37°C in the presence of 5% CO₂. Fresh frozen plasma from healthy individuals was obtained from the blood bank at Lund University Hospital, Lund, Sweden, and kept frozen at -80°C until use.

The synthetic peptides HKH20 (HKHGHGHGKHKNKGKNGKH) and GCP28 (GCPRDIPTNSPELEETLHTITKLNAEN) based on sequences in HK were described previously^{14,15}.

Clotting assays

All clotting times were measured using an Amelung coagulometer. Activated partial thromboplastin time (aPTT) was measured by incubating 30 µl of HKH20 or GCP28 (50 µM final concentration) diluted in sterile water, with 100 µl citrated human plasma for 1 min followed by the addition of 100 µl aPTT reagent (aPTT Automate: Diagnostica Stago, Asnieres, France) for 60 s at 37°C. Clotting was initiated by the addition of 100 µl of a 25 mM CaCl₂ solution. In the Prothombrin time assay (PT) clotting was initiated by the addition of 100 µl Thrombomax with calcium (PT reagent: Sigma-Aldrich). For measuring the Thrombin clotting time (TCT) clotting was initiated by the addition of 100 µl Accuclot thrombin time reagent (TCT reagent: Sigma-Aldrich).

Chromogenic substrate assay

50 ml overnight cultures of *S. pyogenes* AP1 bacteria were washed three times with 50 mM Tris-HCl (pH 7.5), resuspended and diluted to a final concentration of 2 x 10¹⁰ CFU/ml in 50 mM Tris-HCl/ 50 µM ZnCl₂ buffer. Two hundred microliters of bacteria were incubated with 60 µl of HKH20 or GCP28 (final concentration 50 or 100 µM) for 30 s prior to the addition of 200 µl human plasma. Samples were incubated for 30 min at 37°C with shaking. After centrifugation, pellets were washed two times in 50 mM Tris (pH 7,5) centrifuged, resuspended in 100 µl 50 mM Tris-HCl/ 50 µM ZnCl₂ buffer containing 1 mM of the chromogenic substrate S-2302 and incubated for 30 min at 37°C. The samples were centrifuged and the absorbance of the supernatants was measured at 405 nm. No endogenous proteolytic activity was measured when S-2303 was incubated with AP1 bacteria in the absence of plasma.

Bactericidal assay

Bacteria were grown to mid-log phase (OD approximately 0.4 at 620 nm) in TH broth, washed and diluted in 50 mM Tris-HCl (pH 7.5). Fifty microliters of bacteria (2×10^6 CFU/ml) were incubated together with HKH20 at various concentrations for 1 h at 37°C. To quantify the bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, incubated overnight at 37°C, and the number of CFU were determined.

Incubation of bacteria in plasma

Overnight cultures of *S. pyogenes* AP1 bacteria were washed three times with 50 mM Tris-HCl (pH 7.5), resuspended and diluted to a final concentration of 2×10^{10} CFU/ml in 50 mM Tris-HCl/ 50 μ M ZnCl₂ buffer. One hundred microliters of bacteria were incubated with 30 μ l of HKH20 or GCP28 (final concentration 50 or 100 μ M) for 30 s prior to the addition of 100 μ l human plasma. Samples were incubated on a rotator at room temperature for 15 min unless indicated otherwise. After incubation the bacteria were washed two times in 50 mM Tris (pH 7.5), centrifuged, and resuspended in 50 μ l of 50 mM Tris-HCl/ 50 μ M ZnCl₂ buffer. The suspensions were allowed to stay at room temperature for 15 min, followed by centrifugation at 10.000 rpm. Supernatants were collected and kept at -20°C until Western blot analysis.

Electrophoresis and Western blot analysis

SDS-PAGE was performed as described by Neville *et al.* ¹⁶. Proteins in the supernatants from bacterium-plasma incubations were separated on gels of 10% total acrylamide with 3% bisacrylamide. Plasma samples diluted 1/100, untreated or treated with kaolin (Diagnostica Stago, Asmiers, France) for 15 min, served as controls. Before loading, samples were boiled in sample buffer containing 2% (w/v) SDS and 5% (v/v) beta-mercaptoethanol for 10 min. For Western blot analyses, proteins were transferred to nitrocellulose membranes (Immobilon; Millipore), as described previously ¹⁷. Subsequently, nitrocellulose membranes were blocked in phosphate-buffered saline-Tween (PBST) containing 5% (w/v) nonfat dry milk at room temperature for 30 min, washed three times with PBST for 5 min, and incubated with sheep antibodies against HK and its degradation products (1:3000 in the blocking buffer) at room temperature for 60 min. After a washing step, membranes were incubated with peroxidase-conjugated secondary donkey antibodies against goat IgG (1:3000, MP Biomedicals) at room temperature for 60 min. Bound secondary antibodies were detected by the chemiluminescence method

Animal experiments

S. pyogenes AP1 bacteria grown to early logarithmic phase were washed and diluted in PBS to a concentration of 5×10^7 CFU/ml. Female BALB/c mice, 10 – 12 weeks old were injected intraperitoneal (i.p.) with 100 μ l of the bacterial solution, or with 100 μ l of bacteria together with 100 μ l HKH20 (2 mg/ml which corresponds to 200 μ g/mouse) mixed directly before injection, or with 200 μ l PBS alone (control group). Alternatively, mice were treated with 50 μ g aprotinin/mouse (Merck, >5 TIU/mg protein). After 18 h, mice were sacrificed and spleens and lungs were removed. The spleens were kept on ice until homogenization in PBS and the number of CFU in the spleen was quantified by plating serial dilutions of the homogenized material on blood agar plates. Lungs were further processed for microscopic analysis.

For the subcutaneous infection model, mice were anesthetized with isofluorane and injected with 2×10^7 CFU AP1 bacteria in an air-pouch on the neck. Mice showed systemic signs of sickness 8 – 12 hours after infection. To measure clotting times, 8 groups of female BALB/c mice were infected, and after various time points (0 h, 4 h, 6 h, 10 h, 12 h, 18 h, 24 h, and 42 h), the animals were anesthetized with isofluorane and terminal blood samples were taken. Approximately 0.5 ml of blood was drawn by cardiac puncture into polypropylene tubes containing 1/10 volume of 3.8% trisodium citrate. Plasma was separated by centrifugation and clotting times were measured as described earlier. In order to determine bacterial dissemination, spleens were removed and bacterial counts were determined as described above.

PMN depletion was induced by i.p. injection of the anti-mouse Ly-6G (Gr-1,Gr1) antibody (eBioscience, 100 μ g/mouse) 8 hours before infection. Neutropenia was confirmed before starting the infection by manual white blood cell count and FACS analysis¹⁹. To investigate leukocyte recruitment into the peritoneal cavity, mice were sacrificed, 5 ml PBS was injected, and after massage of the peritoneum the fluid was removed and analyzed. Statistical analysis was performed using GraphPadPrism 4.00. The P-value was determined by using the unpaired t-test (comparison of two groups) or the log-rank test (comparison of survival curves). All animal experiments were approved by the regional ethical committee for animal experimentation (permit M209-06).

Pharmacokinetics of HKH20

HKH20 was labeled with the fluorescent dye Alexa Fluor®555 (Molecular Probes) according to the manufacturers protocol. The labeled peptide or the same amount of dye (control) were injected i.p. into mice. Animals were sacrificed after 15, 30, 60 and 120 min. Blood samples were collected and organs (lung, spleen, liver, and kidney)

removed. The fluorescence of the blood samples was measured with a Multilabel counter (Perkin Elmer). Tissue samples were fixed at 4°C for 24 h in buffered 4% formalin (pH 7.4; Kebo), dehydrated and imbedded in paraffin (Histolab Products AB), cut into 4 µm sections, and subjected to fluorescence microscopic analysis. After removal of paraffin, slides were mounted with ProLong®Gold antifade reagent (Invitrogen).

Histochemistry and histo-pathological evaluation

Mice were sacrificed, lungs rapidly removed and fixed at 4°C for 24 h in buffered 4% formalin (pH 7.4; Kebo). Tissues were dehydrated and imbedded in paraffin (Histolab Products AB), cut into 4 µm sections, and mounted. After removal of the paraffin, tissues were stained with Mayers hematoxylin (Histolab Products AB) and eosin (Surgipath Medical Industries, Inc.). Mouse organs were fixed in 4% paraformaldehyde (in PBS, pH 7.4) and processed for routine histo-pathological evaluation.

Scanning Electron Microscopy

Lung samples from the mice were fixed as previously described ²⁰. After fixation, samples were washed, dehydrated, critical point dried, sputtered with palladium/gold as described earlier ²¹. Samples were examined in a Jeol JSM-350 scanning electron microscope.

Results

HKH20 inhibits the intrinsic, but not the extrinsic, pathway of coagulation

Previous studies have shown that domain 5 of HK is a potent adhesin with high affinity for negatively charged surfaces, including cellular membranes (for instance endothelial cells and neutrophils) and artificial substances, such as dextran sulfate or kaolin^{14,22}. Notably, the same domain has also been demonstrated to interact with bacterial surfaces^{23,24}, and subsequent work has led to the identification of the bacteria-binding site in domain 5. A peptide (HKH20) spanning amino acids 479 to 498 of HK was found to mimic the *S. pyogenes* and *Staphylococcus aureus* binding site in HK^{23,24}. It has also been reported that HKH20 can displace HK from other surfaces, including cellular surfaces, such as endothelial cells or synthetic surfaces, such as kaolin or dextran sulfate²⁵. Our findings that radiolabeled HKH20 binds directly to kaolin and *S. pyogenes* (data not shown), is in line with these observations. Concerning the functional activity of HKH20 it should be mentioned that its incubation with dextran sulfate and purified PK, HK, and Factor XII, prevents activation of the contact system²⁶. These properties of HKH20 suggest that it may interfere with the assembly and activation of the contact system *in vitro* and *in vivo*. Initial experiments investigated the ability of HKH20 to inhibit contact activation in plasma and various clotting assays demonstrated that HKH20 impairs the intrinsic pathway of coagulation in normal human plasma and in mouse (BALB/c) plasma. Figure 1a shows that incubation of plasma with HKH20 led to a 4-fold increase of the aPTT when compared with GCP28, a control peptide derived from domain D3 of HK. The effect of HKH20, as depicted in Figure 1b, was dose dependent. In contrast, other parts of the coagulation system as judged by the prothrombin time (PT), monitoring the extrinsic pathway of coagulation, and the thrombin clotting time (TCT), measuring thrombin-induced fibrin-network formation, were not affected in human plasma (Fig. 1c) or in BALB/c plasma (not shown). The results demonstrate that HKH20 exclusively targets the intrinsic pathway of coagulation.

We next investigated whether HKH20 interferes with the activation of PK at negatively charged surfaces. For these experiments kaolin was pre-incubated with HKH20, GCP28, or buffer alone, followed by incubation with human plasma. After 15 min., unbound plasma proteins were removed by a centrifugation and washing step. PK activity at the surface of kaolin was then determined using a specific chromogenic substrate for PK (S-2302, see Material and Methods). As expected, HKH20 efficiently blocks PK activity, whereas the control peptide GCP28 has no influence on the enzymatic activity (Fig. 1d). Since HK is a substrate for activated PK, Western blot

analysis was used to test whether inhibition of PK activity prevents HK degradation. Kaolin was pre-incubated with HKH20 and then mixed with human plasma for 15 min. Plasma alone or plasma treated with kaolin served as negative and positive controls, respectively. Western blots of the diluted samples were stained with antibodies directed against HK and low molecular weight kininogen (LK). Notably, LK is a shorter splice variant of HK²⁷, and a polyclonal antiserum against HK will also react with LK. Figure 1e depicts intact HK at 120kDa (Fig. 1e, lane 1) and processed HK following kaolin treatment (Fig. 1e, lane 2), which triggers the conversion of HK from a single chain to a two chain protein²². When plasma was incubated with kaolin in the presence of HKH20, cleavage of HK was blocked and intact HK was detected (Fig. 1e, lane 3). Taken together, the results demonstrate that HKH20 inhibits kaolin-induced activation of the contact system in human plasma.

HKH20 prevents contact activation at the surface of *S. pyogenes* bacteria

To determine whether treatment with HKH20 inhibits PK activity not only at the surface of kaolin, but also at the surface of *S. pyogenes*, bacteria were incubated with HKH20 followed by the addition of plasma. After 30 min, bacteria were washed and the PK activity at the bacterial surface was determined by measuring hydrolysis of the PK substrate S-2302. The results show that treatment with HKH20 evoked a significant decrease in PK activity as compared to controls incubated with buffer alone or with the GCP28 peptide (Fig. 2a). Next we analyzed whether the co-application of HKH20 prevents HK processing under these experimental conditions. The bacteria were preincubated with HKH20 for 1 min, followed by the addition of plasma for 15 min. They were then washed and resuspended in buffer, followed by an additional incubation step for 15 min to allow the dissociation of bacteria-bound plasma proteins from the streptococcal surface. The proteins in the supernatant were analyzed by a Western blot immunostained with antibodies against HK and LK (Fig. 2b). Non-treated and kaolin-treated plasma served as negative and positive controls, respectively (Fig. 2b, lanes 1 and 2). Western blot analysis of plasma proteins bound to and released from the streptococcal surface revealed that HK was degraded (Fig. 2b, lane 3). However, when bacteria were pre-incubated with 100 μ M HKH20 before plasma was added, the degradation of HK bound to the bacteria was decreased (Fig. 2b, lane 4). As a control, *S. pyogenes* was treated with peptide GCP28, which did not prevent HK cleavage (Fig. 2b, lane 5). The results show that the contact system is assembled and activated at the surface of *S. pyogenes*, and that HKH20 interferes with these molecular events.

HKH20 prevents lung lesions in mice infected with *S. pyogenes* bacteria

The *S. pyogenes* strain used in this study (AP1) belongs to one of the serotypes (M1) that is most frequently associated with severe infections ¹. Unlike most strains of *S. pyogenes*, the AP1 strain is virulent in BALB/c mice ²⁸, which made it possible to study the effect of HKH20 in a mouse model of *S. pyogenes* sepsis. Before testing HKH20 in the sepsis model, we performed toxicity tests to examine potential side effects of the peptide. To this end, 4 mice received intraperitoneal (i.p.) injections (400 µg/dose) of the peptide twice daily for a period of 6 days. Control mice were injected with PBS. Both groups of animals behaved normally, gained weight and appeared completely healthy. Eighteen hours after the final injection, mice were sacrificed and examined. Histo-pathological analysis of heart, lung, spleen, kidney and liver showed normal tissues with no signs of bleeding, blood cell counts and hemograms were also normal (data not shown). Clotting times of plasma samples from HKH20 treated mice were in the same range (aPTT: 36.7 ± 4.1 s, PT: 11.2 ± 0.5 s) as those from the PBS treated mice (aPTT: 35.6 ± 4.5 s; PT: 11.5 ± 0.2 s).

To test the effect of HKH20 in a sepsis model, mice were injected i.p. with 5×10^6 CFU *S. pyogenes*, and different groups (n=5/group) were treated with peptides HKH20, GCP28, or with PBS. Mice injected i.p. with PBS only were used as healthy controls. Eighteen hours after challenge with AP1 bacteria, all infected mice showed clear signs of sickness, such as ruffled fur and less activity. They were sacrificed and lungs were examined by light and scanning electron microscopy. Figure 3 shows representative micrographs of lungs from non-infected mice, displaying no indication of pulmonary damage (Fig. 3a), whereas mice infected with *S. pyogenes* alone (Fig. 3b) or together with peptide GCP28 (Fig. 3d) suffered from severe hemorrhage, alveolar swelling and fibrin deposits. Such lung lesions were almost completely prevented when bacteria were injected together with HKH20 (Fig. 3c). The effect of HKH20 was dose dependent and even when the peptide was given at a lower dose (up to 100 µg/animal) a protective effect was seen (data not shown).

Analysis of the mode of action of HKH20 *in vivo*

Apart from blocking contact activation, HKH20 has also been reported to impair polymorphonuclear neutrophil (PMN) recruitment ²⁵ and to be antibacterial ²⁹. To test whether any of these properties contribute to the protective effect of the peptide, we first investigated the effect of HKH20 on neutrophil influx. Mice were infected i.p. with the bacteria in the presence or absence of HKH20 as described above. Eighteen hours after infection, the animals were sacrificed and leukocyte recruitment into the peritoneum was monitored by FACS analysis and manual counting. Figure 4 shows

that the infection induced a massive invasion of leucocytes, which was not significantly changed when mice were treated with HKH20 ($14.5 \times 10^5 \pm 3.2 \times 10^5$ leucocytes/ml in the untreated group versus $14.7 \times 10^5 \pm 2.7 \times 10^5$ leucocytes/ml in the HKH20 treated group). Injection of HKH20 in uninfected mice did not cause a significant influx of leucocytes (Fig. 4b).

In a next series of experiments we tested whether PMNs contribute to the lung damage in this infection model. Mice were made neutropenic by injecting a monoclonal antibody against a neutrophil surface antigen (Ag GR-1)³⁰, which removed about 97% of all PMNs in the blood of the mice as determined by FACS analysis and white blood cell count (data not shown). Normal and neutropenic mice were infected i.p., in the presence or absence of HKH20 (n=3/group) or, alternatively, challenged with aprotinin, an important inhibitor of the kallikrein/kinin system and other serine proteinases. When left untreated, neutropenic animals developed serious signs of sepsis much faster as compared to normal mice, and the dissemination of bacteria to the spleen also occurred more rapidly. Ten hours after infection all mice (untreated, treated with HKH20 or aprotinin) were sacrificed, lungs and spleens removed, and examined by scanning electron microscopy and bacterial colony counting. As seen before, the bacterial load in spleens of the neutropenic animals was not significantly different, regardless of the treatment (suppl. Fig. 1a). To quantify pulmonary lesions, lung samples from 30 different fields covering an entire lung section were made, and the percentage of the fields exhibiting hemorrhage and fibrin deposits were determined (Fig. 5). When comparing normal infected and neutropenic infected mice in the absence of treatment, we found that lung lesions were reduced from 85 to 61 percent, respectively (Fig. 5a, b), demonstrating that PMN activation and recruitment contributes to, but is not the main cause of the damage. However, a significantly reduced damage was observed when infected mice were treated with HKH20 (Fig. 5c, d) or aprotinin (Fig. 5e, f) in both normal and neutropenic animals. When the lungs were analyzed by light microscopy, similar results were seen (data not shown). A potential antibacterial effect of HKH20 can be excluded since 1) there was no significant difference in the bacterial load of the spleens from normal and neutropenic animals, regardless of HKH20 treatment 2) the concentration of HKH20 used in these experiments was too low to be antibacterial (suppl. Fig. 1b). Taken together, our data suggest that the effect of both substances (HKH20 and aprotinin) relies on the inhibition of contact system activation, rather than preventing PMN activation or recruitment.

To determine the clearance rate of HKH20 we performed a series of experiments in which fluorescently labeled HKH20 (Alexa Fluor®555) was injected i.p. into non-

infected mice (200 µg/animal). Animals were sacrificed after 15, 30, 60, and 120 min and blood and organs (lung, spleen, liver, and kidney) were removed and prepared for further examination. When measuring fluorescence in the plasma samples, we found the highest value 15 min after injection, while after 120 min most of the signal was gone (suppl. Fig. 2). Microscopic analysis of the organs revealed an accumulation of the peptide in lung, spleen, and liver after 15 min, which then decreased within 2h after injection of the peptide, while it appears to accumulate in the kidney within this time (suppl. Fig. 3). These findings suggest that the peptide disseminates evenly in the main organs and is cleared from plasma within 2 hours.

***In vivo* activation of the contact system**

To examine activation of the contact system *in vivo* in an animal model of infection, *S. pyogenes* bacteria were injected subcutaneously (s.c.) in the scruff of the neck³¹ at a dose of *S. pyogenes* causing > 95% mortality. Bacterial dissemination was followed by viable counts of spleen homogenates, and bacteria were earliest detected 10 hours after infection. Infected animals showed severe signs of disease after 18 hours, and death occurred between 24 to 64 hours after infection. To determine when contact activation occurred, plasma samples were collected from infected animals at different time points, and the clotting times of the samples were measured. Figure 6a shows that the aPTT increased in a time dependent manner starting 10 hours after infection. In contrast, the prothombin time was not prolonged during the first 24 hours of infection, but also increased after 42 h (Fig. 6b). Based on these results and considering the clearance rate of HKH20 in plasma, we decided to administer HKH20 eight hours after infection. Electron microscopic analysis of lung biopsies from these animals revealed a significant reduction of lung lesions (Fig. 6c) as compared to infected mice treated with the buffer control (Fig. 6d). In line with these findings, it should be mentioned that infected mice exhibited a time-dependent increase in PK activity (up to 24 h), which was reduced in animals that received HKH20 (data not shown).

The effect of HKH20 treatment on survival

A beneficial effect of HKH20 was also demonstrated in survival studies. Subcutaneously infected mice were treated with a single dose of HKH20 8 hours after infection, and the survival was recorded. Figure 7a shows that 41% of infected mice treated with PBS died during the first 42 hours. In the HKH20 treated group, all animals were alive after 42 hours and statistical analysis revealed that HKH20 treatment caused a significantly prolonged survival time ($P=0.0171$). Comparing the

overall mortality rate of the HKH20 and PBS groups, the HKH20 treated animals showed a tendency toward prolonged survival, but this was not statistically significant ($P=0.0536$) when the experiment was terminated after 96 hours.

Although HKH20 administration resulted in a reversion of *S. pyogenes*–induced lung lesions, and prolonged survival times, the treatment did not significantly affect overall survival. As treatment with HKH20 did not prevent bacterial proliferation, animals might have died because of an overwhelming bacterial load. Thus, in the next series of animal experiments the effect of HKH20 was tested in combination with clindamycin. This administration resembles the clinical situation, since clindamycin is a recommended treatment for patients with invasive streptococcal infection. Initial experiments showed that *S. pyogenes* strain AP1 is clindamycin sensitive (MIC < 0.064 mg/l, E-test). Moreover, when bacteria were grown in plasma together with 10 mg/l clindamycin (156 x MIC conc.) for 24 hours, a 97% reduction of CFUs was recorded. In contrast HKH20 did not affect bacterial growth when added to plasma (220 µg/ml).

To test the effect of HKH20 in combination with clindamycin, mice were subcutaneously infected with *S. pyogenes*. After 18 hours, all animals showed clear signs of advanced sepsis (bacteremia, ruffled fur, average 5% weight loss) and a significant aPTT increase (Fig. 6a). This time point was then also chosen for the first i.p. treatment with clindamycin (10 mg/kg) and HKH20 (200 µg/mouse). Both substances were subsequently administered i.p. at 42, 48 and 72 hours after infection. In the control group mice were injected i.p. with clindamycin (10 mg/kg) only (Fig. 7b). None of the animals treated with clindamycin alone survived, whereas 21% of the animals injected with clindamycin and HKH20 recovered after 168 hours. Statistical analyses revealed a median survival of 48 hours in the clindamycin-treated group versus 97 hours in the clindamycin/HKH20-treated group, which is highly significant ($P<0.0001$). These results show that HKH20 in combination with clindamycin prolongs survival and decreases mortality in mice with severe invasive *S. pyogenes* infection.

Discussion

Sepsis and septic shock constitute a major clinical challenge and despite extensive efforts only one new drug has recently been launched for the treatment of patients suffering from severe infectious diseases. However, this drug, activated protein C (APC), is only recommended in patients at high risk of death (sepsis-induced multiple organ failure, septic shock, or sepsis-induced acute respiratory distress syndrome), and due to the anti-coagulative properties of APC, the protein should not be given to patients at a risk of bleeding. Thus, there is an urgent need for novel therapies with a broader clinical indication and an improved safety profile.

Over the last forty years, the role of the contact system in infectious diseases has attracted considerable attention^{5,32,33}. Several studies have shown that a massive activation of the system can trigger the generation of pathological kinin levels, and lead to a consumption of contact factors followed by impaired hemostasis. For instance, it was demonstrated as early as 1970 by Mason and colleagues that patients with hypotensive septicemia have significantly decreased levels of contact factors³², whereas Pixley and colleagues 1992 published that low levels of FXII and HK in patients with SIRS (systemic inflammatory response syndrome) correlate with a fatal outcome of the disease². These and other findings support the notion that a systemic activation of the contact system contributes to the pathophysiology of severe and invasive infectious diseases (for a review see³⁴).

It is estimated that invasive *S. pyogenes* infections cause more than 150000 deaths annually worldwide³⁵, and the prognosis is especially poor in cases with lung hemorrhage³⁶⁻³⁸. Histological examination of lungs recovered from patients who died from fulminant *S. pyogenes* infection revealed severe intra-alveolar hemorrhage³⁸, and in these patients the aPTT was dramatically prolonged³⁸. Similar findings were observed in the present study when lungs and plasma samples from infected mice were analyzed. This implies that the animal model used here mimics these fulminant *S. pyogenes* infections in humans. Our results show that the lung lesions in the infected mice are prevented when animals are treated with a contact system inhibitor. However, they also suggest that neutrophils play a role in this process, since lung lesions decreased from 85% to 61%, respectively, in neutropenic mice (Fig. 5a+b). This is in concordance with previous studies showing that M1 protein, a classic virulence determinant of *S. pyogenes*, is able to activate neutrophils and cause lung lesions in mice^{31,39}. It was also demonstrated in a *S. pyogenes* infection model, that pulmonary damage was prevented when neutrophil activation was blocked³¹. However, in that study the inhibitor was given before the infection became invasive (30 min) and the mice were sacrificed after 6h, implying that the contact system was

not activated at that stage. These findings may also explain the present results that HKH20 treatment of infected mice at an earlier time point (2 h after s.c. infection) and before the contact system was activated, had no beneficial effect.

HKH20 is derived from a region of HK responsible for binding to bacterial and eukaryotic cell surfaces ^{14,23}. Interference with this binding was found to block the assembly and activation of the system *in vitro*. In previous studies, we used an irreversible contact system inhibitor to prevent severe lung lesions in a rat model of *Salmonella* sepsis ⁴⁰. This tri-peptide derivative (H-D-Pro-Phe-Arg-CMK) forms a covalent link with the catalytic pocket of PK and FXII ^{41,42}. As the CMK-group (chloromethylketone) is toxic and the substance may also inhibit other serine proteases when given at therapeutic doses, it is an unrealistic drug candidate. HKH20, on the other hand, has a completely different mode of action. The peptide displaces HK from its binding to negatively charged surfaces but does not influence the enzymatic activity of PK and FXII. The interference with a defined protein interaction should also enhance the specificity of HKH20. Moreover, HKH20 is not cytotoxic ²⁹, and the analysis in the present toxicity study showed that the peptide is well tolerated, also when administered to mice in doses much higher than required for a therapeutic effect. No signs of bleeding disorders in the organs were observed in the animals. In contrast to APC that interferes with the extrinsic and the primary pathway of coagulation, HKH20 blocks the intrinsic system, which plays a secondary role in hemostasis. This and the fact that the peptide prevents lung bleedings and tissue damage, and, in combination with clindamycin, prolonged survival time and increased overall survival, could represent a novel therapeutic principle in severe infectious diseases.

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Authorship

S. Oehmcke performed research, analyzed the data and wrote the paper, O. Shannon performed research, M. v. Köckritz-Blickwede contributed analytic tools, M. Mörgelin contributed analytic tools and performed research, A. Linder performed research, A. I. Olin performed research, L. Björck designed research and wrote the paper, H. Herwald designed research and wrote the paper.

References:

1. Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev. 2000;13:470-511.
2. Pixley RA, Zellis S, Bankes P, et al. Prognostic value of assessing contact system activation and factor V in systemic inflammatory response syndrome. Crit Care Med. 1995;23:41-51.
3. Ben Nasr A, Herwald H, Sjöbring U, Renné T, Müller-Esterl W, Björck L. Absorption of kininogen from human plasma by *Streptococcus pyogenes* is followed by the release of bradykinin. Biochem J. 1997;326 (Pt 3):657-660.
4. Bhoola KD, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol Rev. 1992;44:1-80.
5. Colman RW. The contact system and sepsis. Prog Clin Biol Res. 1994;388:195-214.
6. Colman RW, Schmaier AH. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. Blood. 1997;90:3819-3843.
7. Frick IM, Akesson P, Herwald H, et al. The contact system-a novel branch of innate immunity generating antibacterial peptides. EMBO J. 2006;25:5569-5578.
8. Herwald H, Mörgelin M, Olsen A, et al. Activation of the contact-phase system on bacterial surfaces-a clue to serious complications in infectious diseases. Nat Med. 1998;4:298-302.
9. Persson K, Mörgelin M, Lindbom L, Alm P, Björck L, Herwald H. Severe lung lesions caused by *Salmonella* are prevented by inhibition of the contact system. J Exp Med. 2000;192:1415-1424.
10. Pixley RA, De La Cadena R, Page JD, et al. The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal bacteremia. *In vivo* use of a monoclonal anti-factor XII antibody to block contact activation in baboons. J Clin Invest. 1993;91:61-68.
11. Pixley RA, DeLa Cadena RA, Page JD, et al. Activation of the contact system in lethal hypotensive bacteremia in a baboon model. Am J Pathol. 1992;140:897-906.
12. Sriskandan S, Kembell-Cook G, Moyes D, Canvin J, Tuddenham E, Cohen J. Contact activation in shock caused by invasive group A *Streptococcus pyogenes*. Crit Care Med. 2000;28:3684-3691.
13. von Köckritz-Blickwede M, Rohde M, Oehmcke S, et al. Immunological Mechanisms Underlying the Genetic Predisposition to Severe Staphylococcus aureus Infection in the Mouse Model. Am J Pathol. 2008.
14. Hasan AA, Cines DB, Herwald H, Schmaier AH, Müller-Esterl W. Mapping the cell binding site on high molecular weight kininogen domain 5. J Biol Chem. 1995;270:19256-19261.
15. Herwald H, Hasan AA, Godovac-Zimmermann J, Schmaier AH, Müller-Esterl W. Identification of an endothelial cell binding site on kininogen domain D3. J Biol Chem. 1995;270:14634-14642.
16. Neville DM, Jr. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J Biol Chem. 1971;246:6328-6334.
17. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979;76:4350-4354.
18. Nesbitt SA, Horton MA. A nonradioactive biochemical characterization of membrane proteins using enhanced chemiluminescence. Anal Biochem. 1992;206:267-272.
19. Shannon O, Herten E, Norrby-Teglund A, Mörgelin M, Sjöbring U, Björck L. Severe streptococcal infection is associated with M protein-induced platelet activation and thrombus formation. Mol Microbiol. 2007;65:1147-1157.

20. Herwald H, Mörgelin M, Dählbäck B, Björck L. Interactions between surface proteins of *Streptococcus pyogenes* and coagulation factors modulate clotting of human plasma. *J Thromb Haemost.* 2003;1:284-291.
21. Oehmcke S, Mörgelin M, Herwald H. Activation of the human contact system on neutrophil extracellular traps. *J Innate Immun.* 2009;225 - 230.
22. Herwald H, Mörgelin M, Svensson HG, Sjöbring U. Zinc-dependent conformational changes in domain D5 of high molecular mass kininogen modulate contact activation. *Eur J Biochem.* 2001;268:396-404.
23. Ben Nasr AB, Herwald H, Müller-Esterl W, Björck L. Human kininogens interact with M protein, a bacterial surface protein and virulence determinant. *Biochem J.* 1995;305 (Pt 1):173-180.
24. Mattsson E, Herwald H, Cramer H, Persson K, Sjöbring U, Björck L. *Staphylococcus aureus* induces release of bradykinin in human plasma. *Infect Immun.* 2001;69:3877-3882.
25. Chavakis T, Kanse SM, Pixley RA, et al. Regulation of leukocyte recruitment by polypeptides derived from high molecular weight kininogen. *Faseb J.* 2001;15:2365-2376.
26. Nakazawa Y, Joseph K, Kaplan AP. Inhibition of contact activation by a kininogen peptide (HKH20) derived from domain 5. *Int Immunopharmacol.* 2002;2:1875-1885.
27. Furuto-Kato S, Matsumoto A, Kitamura N, Nakanishi S. Primary structures of the mRNAs encoding the rat precursors for bradykinin and T-kinin. Structural relationship of kininogens with major acute phase protein and alpha 1-cysteine proteinase inhibitor. *J Biol Chem.* 1985;260:12054-12059.
28. Björck L, Åkesson P, Bohus M, et al. Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. *Nature.* 1989;337:385-386.
29. Nordahl EA, Rydengård V, Mörgelin M, Schmidtchen A. Domain 5 of high molecular weight kininogen is antibacterial. *J Biol Chem.* 2005;280:34832-34839.
30. Conlan JW, North RJ. Neutrophils are essential for early anti-*Listeria* defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. *J Exp Med.* 1994;179:259-268.
31. Herwald H, Cramer H, Mörgelin M, et al. M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell.* 2004;116:367-379.
32. Mason JW, Kleeberg U, Dolan P, Colman RW. Plasma kallikrein and Hageman factor in Gram-negative bacteremia. *Ann Intern Med.* 1970;73:545-551.
33. Frick IM, Björck L, Herwald H. The dual role of the contact system in bacterial infectious disease. *Thromb Haemost.* 2007;98:497-502.
34. Pixley RA, Colman RW. The kallikrein-kinin system in sepsis syndrome. *Handbook of Immunopharmacology - The Kinin System* Farmer SG, ed New York: Academic Press. 1997:173-186.
35. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. *Lancet Infect Dis.* 2005;5:685-694.
36. Barnham M, Weightman N, Anderson A, Pagan F, Chapman S. Review of 17 cases of pneumonia caused by *Streptococcus pyogenes*. *Eur J Clin Microbiol Infect Dis.* 1999;18:506-509.
37. Montgomery VL, Bratcher D. Complications associated with severe invasive streptococcal syndrome. *J Pediatr.* 1996;129:602-604.
38. Ooe K, Nakada H, Udagawa H, Shimizu Y. Severe pulmonary hemorrhage in patients with serious group A streptococcal infections: report of two cases. *Clin Infect Dis.* 1999;28:1317-1319.
39. Soehnlein O, Oehmcke S, Ma X, et al. Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein. *Eur Respir J.* 2008;32:405-412.

40. Persson K, Mörgelin M, Lindbom L, Alm P, Björck L, Herwald H. Severe lung lesions caused by Salmonella are prevented by inhibition of the contact system. *J Exp Med.* 2000;192:1415-1424.
41. Ghebrehiwet B, Randazzo BP, Dunn JT, Silverberg M, Kaplan AP. Mechanisms of activation of the classical pathway of complement by Hageman factor fragment. *J Clin Invest.* 1983;71:1450-1456.
42. Tans G, Janssen-Claessen T, Rosing J, Griffin JH. Studies on the effect of serine protease inhibitors on activated contact factors. Application in amidolytic assays for factor XIIa, plasma kallikrein and factor XIa. *Eur J Biochem.* 1987;164:637-642.

Figure legends

Fig. 1: HKH20 interferes with the intrinsic pathway of coagulation.

a) Human plasma or BALB/c mouse plasma was incubated with 50 μ M HKH20, GCP28, or buffer alone (control) for 60 s and analyzed by the aPTT test. b) Human plasma was incubated with increasing amounts of HKH20 or GCP28 and the aPTT was measured. c) Normal human plasma was incubated with 50 μ M HKH20, GCP28 or buffer alone (control) for 60 s and analyzed by the PT and the TCT tests. d) Human plasma was incubated with kaolin in the presence of 50 μ M HKH20, GCP28 or buffer alone (control) for 15 min. Plasma was removed by centrifugation and pelleted kaolin was washed and resuspended in substrate buffer. After 15 min of incubation, plasma kallikrein activity was measured in a substrate assay. Data are presented as percent activity compared to the control, values are means $\pm$ standard deviations (n=3). e) Human plasma was incubated with buffer (lane 1), kaolin (lane 2), or kaolin and 50 μ M HKH20 (lane 3) for 15 min. Samples were analyzed by Western blotting with antibodies identifying HK and LK.

Fig. 2: HKH20 inhibits *S. pyogenes*-induced contact activation

a) Human plasma was incubated with *S. pyogenes* bacteria in the presence of 50 μ M HKH20, GCP28, or buffer alone (control) for 30 min. Plasma was removed by centrifugation and bacteria were washed and resuspended in substrate buffer. After 30 min of incubation, plasma kallikrein activity was measured in a substrate assay. Data are presented as percent activity compared to the control, values are mean $\pm$ SD (n=3, *p < 0.05 by t test). b) Human plasma was incubated with *S. pyogenes* bacteria in the presence or absence of HKH20 or GCP28 for 15 min. Bacteria were washed, resuspended in buffer, incubated for 15 min and spun down. Supernatants and plasma (non-treated or kaolin-treated) were analyzed by SDS PAGE and Western blotting with antibodies against HK and LK, Lanes: 1) normal plasma, 2) kaolin-treated plasma, 3) plasma proteins absorbed and released by *S. pyogenes*, 4) plasma proteins absorbed and released by *S. pyogenes* in the presence of 100 μ M HKH20, 5) plasma proteins absorbed and released by *S. pyogenes* in the presence of 100 μ M GCP28. Vertical lines have been inserted to indicate a repositioned gel lane.

Fig. 3: HKH20 prevents lung damage in BALB/c mice infected i.p. with *S. pyogenes*

Light microscopy (left) and scanning electron microscopy (right) of representative mouse lung tissue sections are shown. Mice were injected i.p. a) with 200 μ l PBS, b) 5×10^6 CFU *S. pyogenes*, c) 5×10^6 CFU *S. pyogenes* and 200 μ g HKH20, and d) 5×10^6 CFU *S. pyogenes* and 275 μ g GCP28. Bars represent 250 μ m (light microscopy) and 50 μ m (scanning electron microscopy).

Fig. 4: Leukocyte recruitment is not impaired by HKH20

FACS analysis of peritoneal lavage from non-infected mice injected with PBS (a) or HKH20 (b), mice infected i.p. with 5×10^6 CFU *S. pyogenes* in the absence (c) or presence of 200 μ g HKH20 (d). Peritoneal lavage was analyzed 18 hours after infection. All leucocytes are CD45 positive and neutrophil populations (red) are separated from monocyte populations (green) based on their side scatter pattern.

Fig. 5: Comparison of lung lesions in normal and neutropenic mice infected with *S. pyogenes*

Scanning electron micrographs of representative mouse lung tissue sections are shown. Normal (a, c, e) or neutropenic (b, d, f) mice were injected i.p. with a, b) 5×10^6 CFU *S. pyogenes*, c, d) 5×10^6 CFU *S. pyogenes* and 200 μ g HKH20 e, f) 5×10^6 CFU *S. pyogenes* and 50 μ g aprotinin. Bar represents 50 μ m.

Fig. 6: Contact activation *in vivo* and treatment with HKH20

Mice were injected subcutaneously in the neck with 2×10^7 CFU *S. pyogenes* bacteria, plasma was collected at 0, 4, 6, 10, 12, 18, 24, and 42 hours after infection (n=2-6/group), and aPTT (a) and PT (b) were measured immediately (*P<0.05; ***P<0.0001). c) Scanning electron microscopy of representative mouse lung tissue sections are shown. Mice were injected s.c. with 2×10^7 CFU *S. pyogenes* and treated i.p. 8 hours after infection with 200 μ g HKH20 (c) or 100 μ l PBS (d). Lungs were taken 18 hours after infection, the bar represents 100 μ m.

Fig. 7: HKH20 improves survival in a mouse model of *S. pyogenes* sepsis

a) Mice were injected s.c. in the neck with 2×10^7 CFU *S. pyogenes* bacteria and treated with 100 μ l HKH20 (200 μ g; ●) or 100 μ l PBS (■) i.p. 8 hours after infection (n=4-5/group). Mortality was recorded for a period of 5 days. The experiment was repeated four times and the results from a total of 17 animals per group are shown. b) Mice were infected s.c. with 2×10^7 CFU *S. pyogenes* bacteria and treated with 10 mg/kg clindamycin (▲) or 200 μ g HKH20 and 10 mg/kg clindamycin (●) in a volume

of 200 µl PBS. Treatment was i.p. injection at 18, 42, 48 and 72 hours after infection (n=5/group). Mortality was recorded for a period of 7 days. The experiment was repeated three times and the results from a total of 15 animals per group are shown.

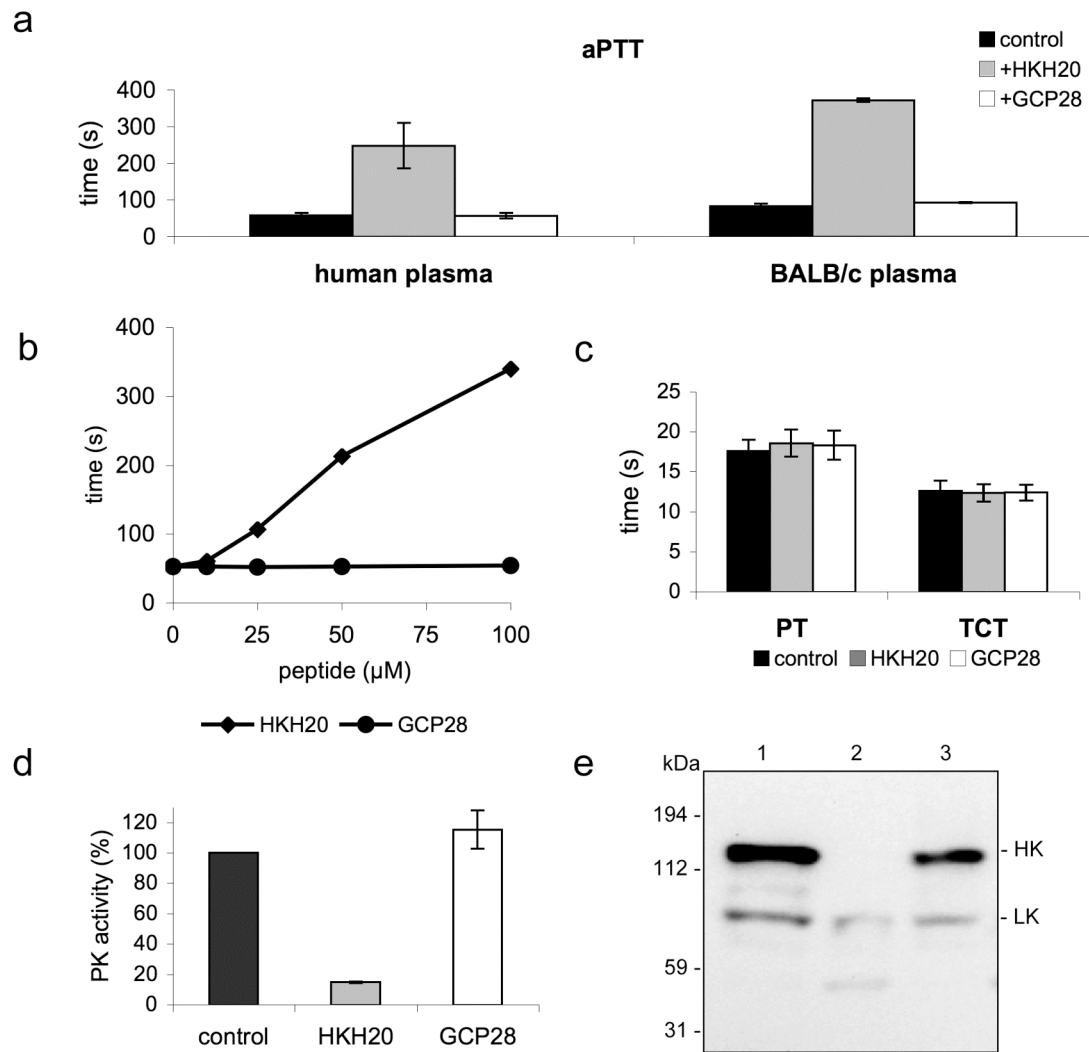


Fig. 1

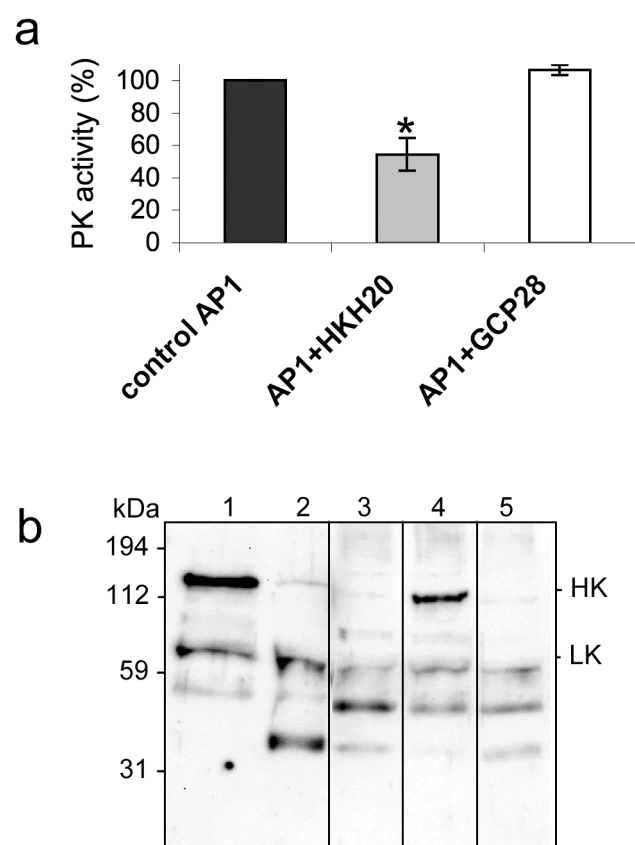


Fig. 2

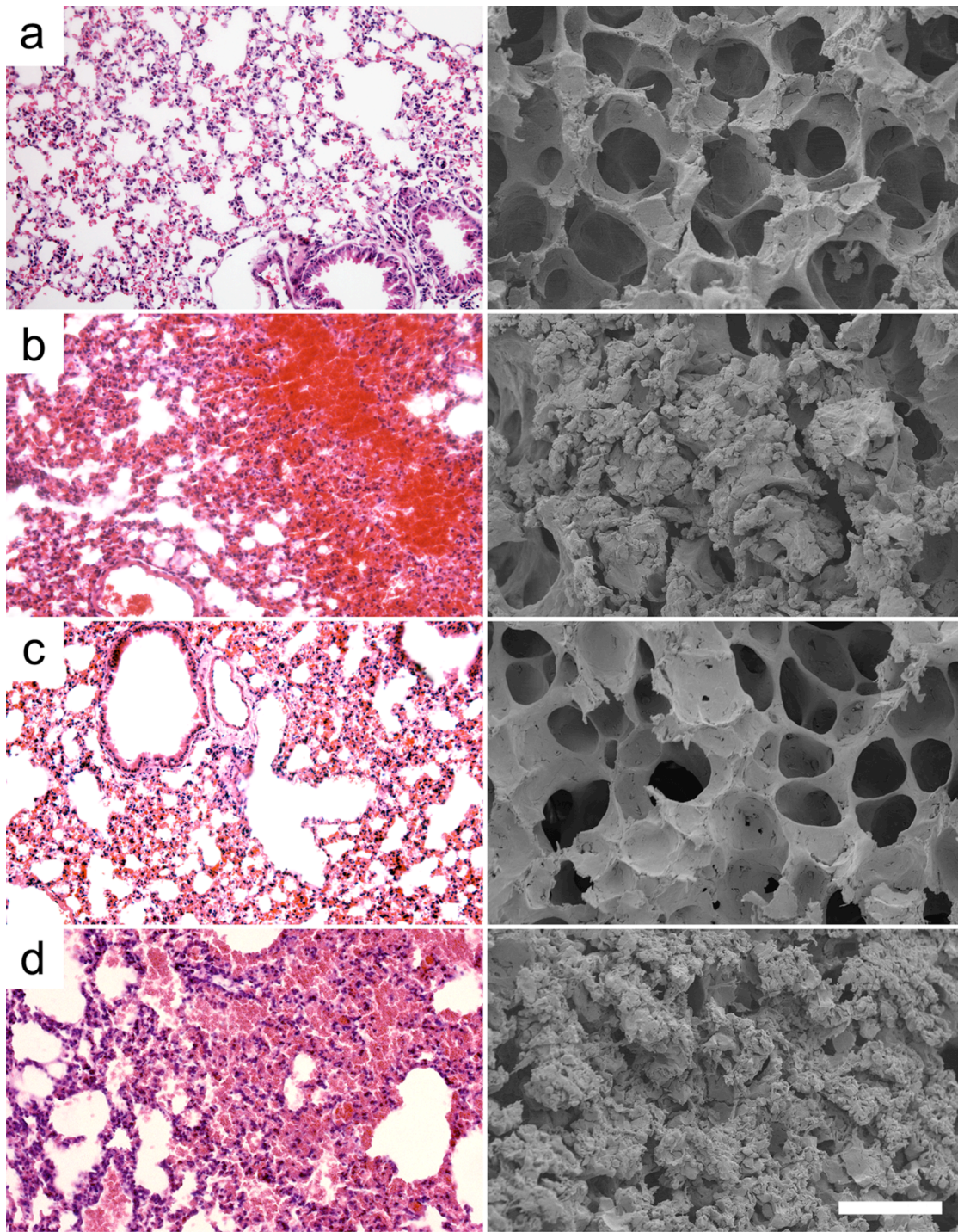


Fig. 3

Fig. 3

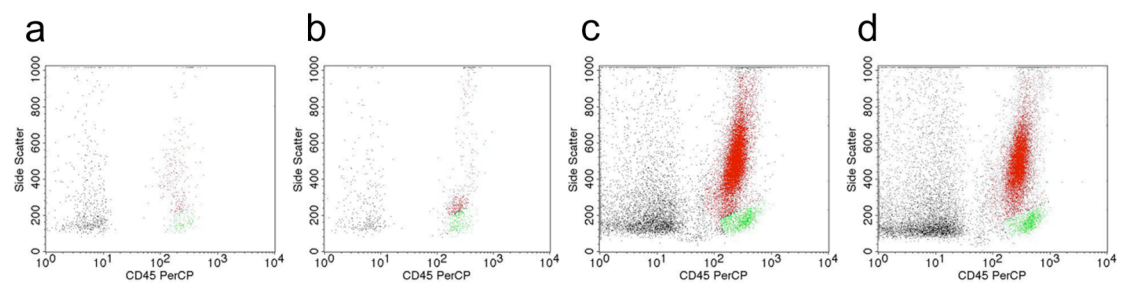


Fig. 4

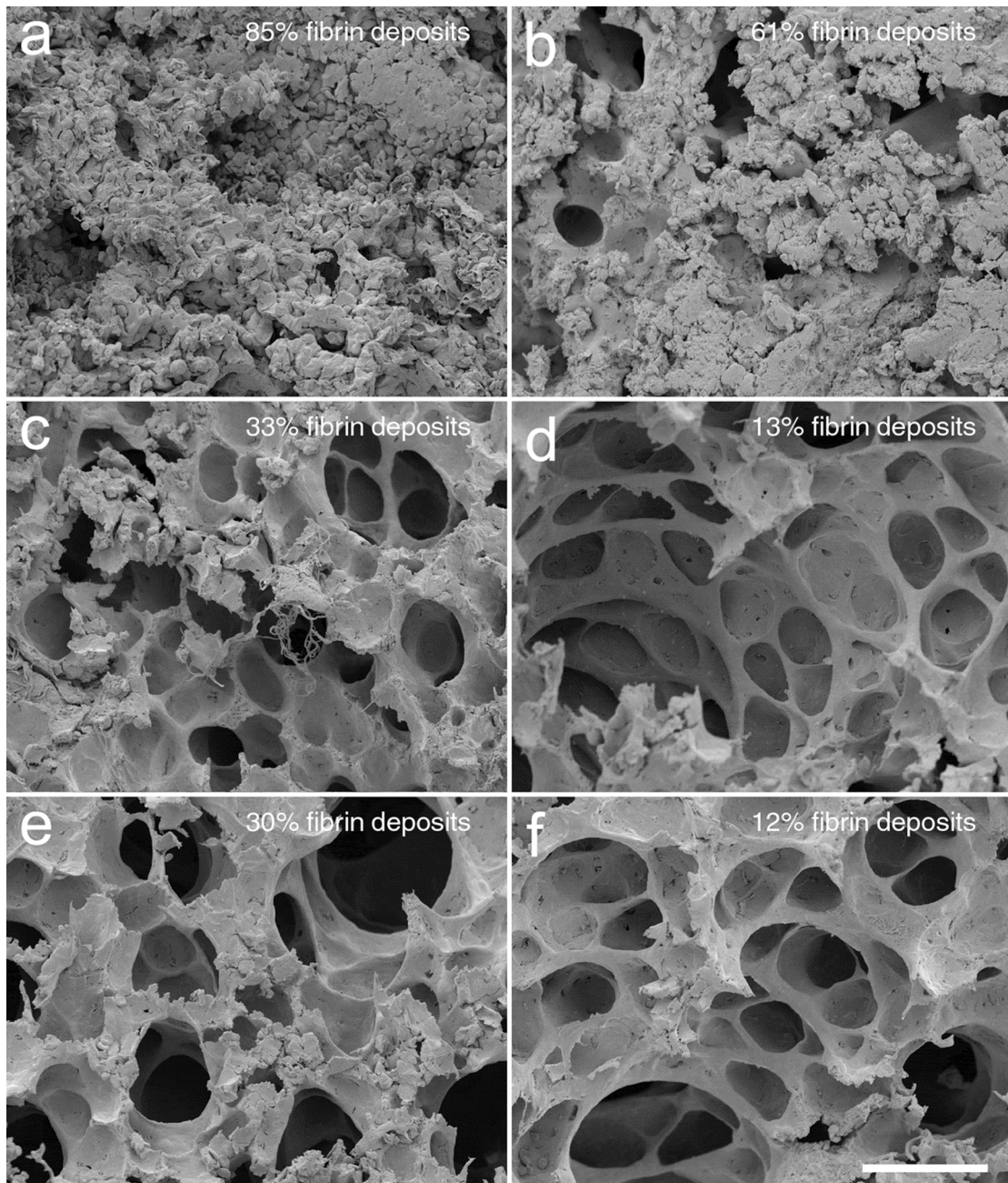


Fig. 5

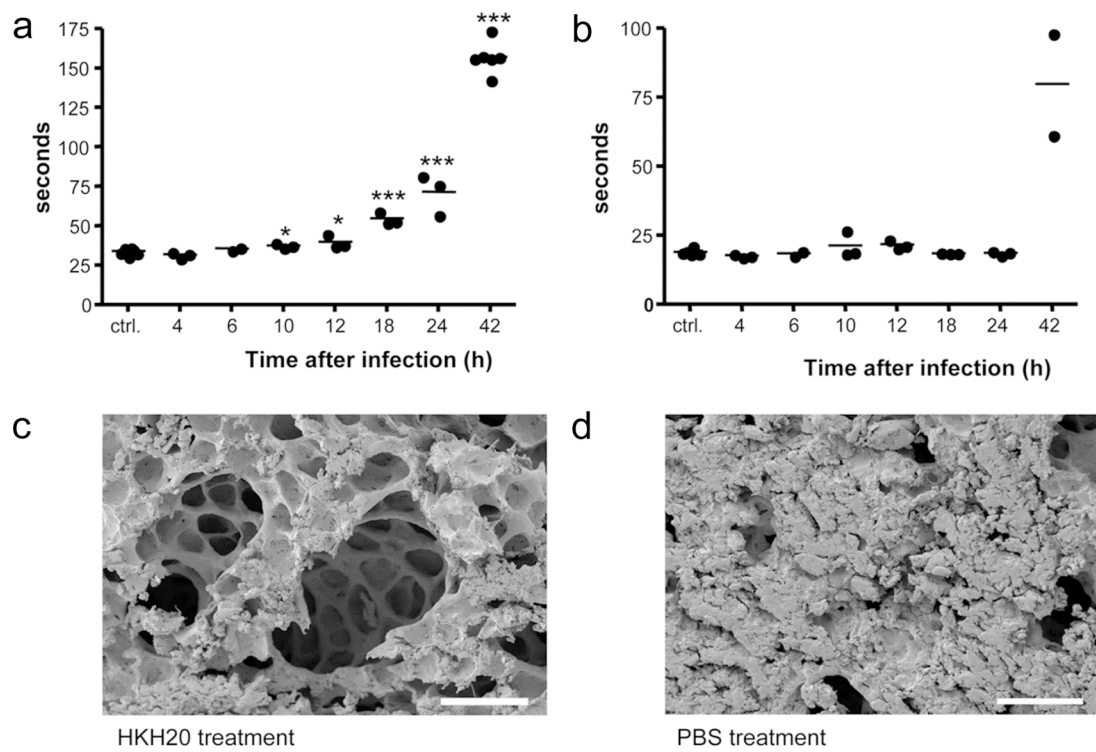


Fig. 6

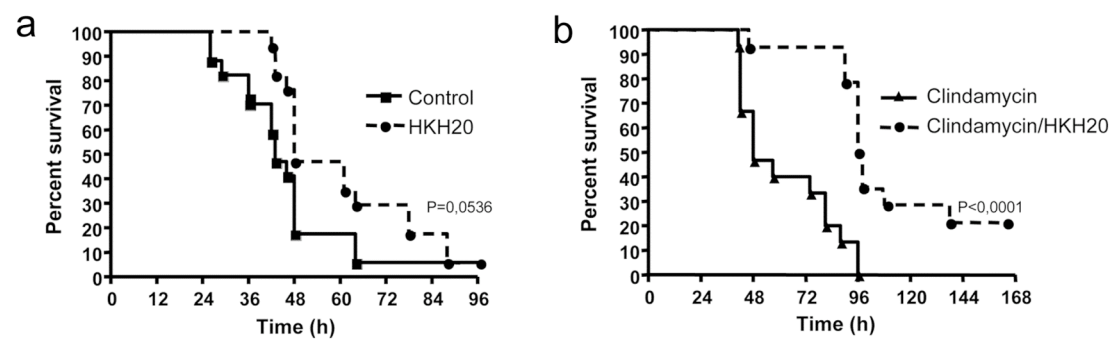


Fig. 7