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Mechanismen der Kontaktaktivierung während einer Infektion mit *Streptococcus pyogenes*

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Inhaltsverzeichnis

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2. Einleitung

2.1 *Streptococcus pyogenes*

Streptococcus pyogenes ist ein grampositives, kokkenförmiges, in der Regel in Ketten angeordnetes Bakterium der Familie der *Streptococcaceae* aus der Ordnung der *Lactobacillales* und der Klasse der *Bacilli*. Die Bacilli werden dem Phylum der *Firmicutes* zugeordnet.

Die Familie der *Streptococcaceae* wird anhand eines immunogenen Oberflächen-Polysaccharides in die Gruppen A bis W eingeteilt. Nach dieser Typisierung ist *S. pyogenes* der alleinige Vertreter der Gruppe A und wird daher auch als Gruppe A Streptococcus (= GAS) bezeichnet (Lancefield, 1933).

S. pyogenes ist ein bedeutendes humanpathogenes Bakterium welches vornehmlich Haut- und Schleimhautinfektionen verursacht, wie z.B. Erysipel oder Tonsillitis, aber auch Toxin-assoziierte Infektionskrankheiten wie Scharlach. Diese Infektionen sind normalerweise lokal begrenzt und selbstlimitierend, können aber gelegentlich zu schweren systemischen oder

invasiven Verläufen mit lebensbedrohlichen Komplikationen führen, wie dem Streptokokken-assoziiertem *Toxic-Shock-like* Syndrome (STSS) und die nekrotisierende Fasziitis. Bei fehlender oder verspäteter antibiotischer Behandlung sind außerdem Autoimmun-Folgeerkrankungen möglich. Zu diesen gehören die akute Glomerulonephritis und das akute rheumatische Fieber (Cunningham, 2000; Walker *et al*, 2014). Insgesamt verursacht *S. pyogenes* jährlich weltweit etwa 111 Millionen Fälle von Pyodermien, mehr als 616 Millionen neue Fälle von Pharyngitis und etwa 500.000 Todesfälle aufgrund schwerer invasiver Erkrankungen. *S. pyogenes* gehört damit, neben dem HIV- oder Tuberkulose-Erregern, zu den 10 wichtigsten Pathogenen weltweit (Carapetis *et al*, 2005).

Während der Pathogenese exprimiert *S. pyogenes* eine große Vielfalt an Virulenzfaktoren, welche mit humanen Strukturen interagieren um z.B. die Kolonisierung und das Eindringen in Epithelzellen zu ermöglichen. Weiterhin werden sie benötigt um dem Immunsystem des Wirtes zu entkommen, im Blut zu überleben und sich in andere Organe auszubreiten. Ihre Zusammensetzung und Expression erfolgt in Abhängigkeit der Wachstumsphase und des entsprechenden Serotypen. Bis heute sind etwa 50 verschiedene Virulenzfaktoren beschrieben (Kreikemeyer *et al*, 2003).

Ein wichtiger Virulenzfaktor ist das Zellwand-assoziierte immunogene M-Protein. Dieses oberflächen-assoziierte Protein besitzt ein hypervariables aminoterminales Ende, welches zur Serotypisierung von bislang 100 verschiedenen Serotypen genutzt wurde (Kotloff & Dale, 2004). Die Typisierung erfolgt heute allerdings in der Regel nicht mehr serologisch, sondern mittels Sequenzierung des M-Protein-kodierenden *emm* Gens (Podbielski *et al*, 1991), wodurch zurzeit mehr als 200 *emm* Typen unterschieden werden (Sanderson-Smith *et al*, 2014).

M-Proteine sind in Adhärenz, Kolonisierung und Internalisierung involviert, schützen vor Phagozytose sowie gegen antimikrobelle Peptide, triggern inflammatorische Reaktionen und intervenieren mit dem humanen Gerinnungs- und Kontaktsystem (siehe Review Oehmcke *et al*, 2010).

2.2 Das humane Gerinnungs- und Kontaktsystem

Das Gerinnungssystem des Menschen ist essentieller Teil der Hämostase, ein Prozess der den Stillstand einer Blutung herbeiführt. Dies ist nicht nur wichtig um sich bei einer Verletzung vor Blutverlust zu schützen, sondern auch um eine Barriere gegen eindringende Bakterien aufzubauen (Macrae *et al*, 2018). Die Hämostase ist somit ein wichtiger Abgrenzungs- und Abwehrmechanismus, und die lokale Aktivierung der Gerinnung zählt heute zu einem wichtigen angeborenen Immunmechanismus des Menschen (van der Poll & Herwald, 2014).

Man unterscheidet zwischen primärer und sekundärer Hämostase. Bei der primären Hämostase kommt es zunächst zu einer Vasokonstriktion des verletzten Gefäßes und zu einer Adhäsion und Aktivierung von Thrombozyten an der verletzten Gefäßstelle. Durch die nachfolgende sekundäre Hämostase erfolgt die eigentliche Blutgerinnung, d.h. es bildet sich ein festes Netz aus Fibrinfasern, welches den Thrombus stabilisiert. Die Fibrinbildung kann durch 2 unterschiedliche Mechanismen initiiert werden: durch Bestandteile der verletzten Gefäßwand (extrinsisches System) oder durch Komponenten des Plasmas (intrinsisches System).

Das intrinsische System wird auch als Kontaktsystem bezeichnet, denn wenn Blut in Kontakt mit negativ geladenen artifiziellen Oberflächen gerät, werden die Faktoren des Kontaktsystems aktiviert (Abbildung 1). Das System besteht aus 2 Serinproteasen, Faktor XII (FXII) und Plasmakallikrein (PK) sowie hochmolekularem Kininogen (HK), einem nicht-enzymatische Kofaktor (siehe Abbildung 1). Die Proteine werden in der Leber gebildet und zirkulieren als Zymogene im Blut oder sind an der Oberfläche von Endothelzellen, Neutrophilen und Thrombozyten assembliert. Wenn das Blut mit einer artifiziellen Oberfläche in Kontakt gerät, bindet FXII daran, verändert dadurch seine Konformation und wird durch Autoaktivierung zum aktiven Gerinnungsfaktor FXIIa (Samuel *et al*, 1992). Klassischerweise wurde deklariert, dass FXII durch negativ geladene Oberflächen aktiviert wird. Heute geht man davon aus das jede artifizielle Oberfläche das Potential zur Autoaktivierung von FXII besitzt (Schmaier, 2016). HK, welches im Plasma in einem nicht-kovalenten Komplex mit Pre-Plasmakallikrein (PPK) vorliegt (Mandle *et al*, 1976), bindet ebenfalls an die Oberfläche und präsentiert dadurch PPK für die Aktivierung durch FXIIa. Dieser aktiviert PPK zu PK, welches in einem positiven Rückkopplungsmechanismus weiteren FXIIa produziert. Wenn genügend FXIIa vorliegt startet dieser durch Aktivierung von Faktor XI (FXI) die Gerinnungskaskade, was letztendlich zur Bildung von Thrombin führt. Dies resultiert - *in vitro* - in der Bildung eines Fibrinnetzwerkes, und deshalb wird dieser Teil des Kontaktsystems als der prokoagulante Teil beschrieben.

PK kann aber, neben seiner Wirkung auf FXII, aus HK das Peptidhormon Bradykinin freisetzen (Colman & Schmaier, 1997). Bradykinin ist einer der potentesten inflammatorischen Mediatoren des Menschen, es bindet an zelluläre Kinin-B2-Rezeptoren und aktiviert proinflammatorische Signalwege (Leeb-Lundberg *et al*, 2005). Dieser Weg des Kontaktsystems – das sog. Kallikrein-Kinin System (siehe Abbildung 1) - wird deshalb auch als der proinflammatorische Teil beschrieben.

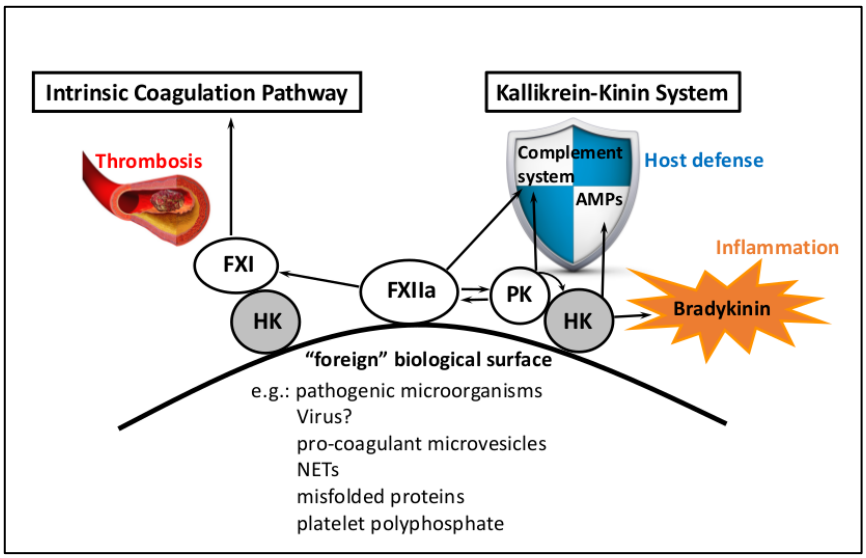

Abbildung 1: Das humane Kontaktsystem. Bindung und Assemblierung von Kontaktfaktoren an biologische oder artifizielle Oberflächen aktiviert FXII. FXIIa aktiviert FXI welcher den intrinsischen Gerinnungsweg triggert. FXIIa aktiviert auch PK, dieses schneidet HK wobei Bradykinin und antimikrobielle Peptide freigesetzt werden. Außerdem können FXII und PK *in vitro* Bestandteile des Komplementsystems aktivieren. Übernommen aus (Oehmcke-Hecht & Köhler, 2018).

Die Autoaktivierung von FXII bei Kontakt mit artifiziellen Oberflächen ist Basis des Gerinnungstests „Aktivierte partielle Thromboplastinzeit“ (aPTT). Personen mit einem angeborenen Mangel an den Kontaktfaktoren FXII, PK oder HK, haben eine deutlich verlängerte aPTT, aber keine Neigung zu Blutungen oder beeinträchtigter Blutstillung, so wie dass der Fall bei z.B. angeborenen FVIII Mangel ist. Die Fibrinbildung über den prokoagulanten Teil des Kontaktsystems scheint somit keine essentielle Rolle bei der physiologischen Hämostase zu spielen (Meijers, 2014). Darüber hinaus findet man eine systemische Kontaktaktivierung ausschließlich bei pathologischen Zuständen, wie z.B. Thrombose (Renné *et al*, 2012), Sepsis oder akutem respiratorischen Distress Syndrom (ARDS) (Hess *et al*, 2017; Oehmcke & Herwald, 2010a), was FXII zu einem attraktiven therapeutischen Target macht (Long *et al*, 2016). Es ist also fraglich ob die Gerinnungsaktivierung durch FXII *in vivo* wirklich eine physiologische Rolle spielt. Stattdessen geht man heute davon aus, dass der proinflammatorische Teil des Systems eher eine physiologische *in vivo* Funktion einnimmt (Jukema *et al*, 2016). Die Aktivierung von Kontaktfaktoren während einer Infektion triggert inflammatorische Reaktionen, die die Immunantwort gegen eindringende Pathogene potenzieren (siehe Review Oehmcke-Hecht & Köhler, 2018).

Die Hämostase wird durch das eng regulierte plasmatische Gerinnungssystem aufrechterhalten. Während einer Infektion wird das Gerinnungssystem aktiviert und trägt zur angeborenen Immunantwort bei, indem das Eindringen und die Ausbreitung von Bakterien verhindert wird. *S. pyogenes* hat verschiedene Strategien entwickelt diese lokale

Immunantwort zu umgehen. Auf der anderen Seite sind diese Bakterien in der Lage Gerinnungs- und Kontaktsystem massiv zu aktivieren, was im Wirt großen Schaden anrichtet (Shannon *et al*, 2013). Insbesondere im Verlauf von schwerwiegenden *S. pyogenes* Infektionen wie STSS oder Sepsis kommt es zu lebensbedrohlichen Gerinnungsstörungen, wobei die molekularen Mechanismen der Pathogenese immer noch nicht hinreichend verstanden sind.

Ziel dieser Arbeit war es solche Mechanismen näher zu untersuchen um neue Behandlungsstrategien für lebensbedrohliche Infektionen entwickeln zu können.

3. Ergebnisse und Diskussion

3.1 Ein Peptid aus hochmolekularem Kininogen (HK) zur Behandlung von invasiven Streptokokken Infektionen (Anhang 1)

Sepsis und septischer Schock sind Komplikationen bakterieller Infektionen die, ungeachtet einer antibiotischen und intensivmedizinischen Behandlung, mit einer hohen Mortalitätsrate einhergehen. Sepsis ist weiterhin das archetypische Krankheitsbild, bei dem eine systemische Kontaktaktivierung stattfindet (Schmaier, 2014).

In früheren Studien wurde gezeigt, dass *S. pyogenes* alle Kontaktfaktoren an seiner Oberfläche bindet, was zu einer Aktivierung des Systems mit Freisetzung von Bradykinin führt (Ben Nasr *et al*, 1997). Das M-Protein an der Oberfläche der Bakterien spielt dabei eine wichtige Rolle für die Bindung von HK (Ben Nasr *et al*, 1995). Bradykinin ist ein potenter inflammatorischer Mediator. Es induziert eine erhöhte vaskuläre Durchlässigkeit mit kapillarer Leckage, was in der Folge mit Ödembildung, Schmerzen und Hypotension einhergehen kann (Bhoola *et al*, 1992). Eine Kontaktsystemaktivierung an der Oberfläche der Bakterien, welche durch Bradykininfreisetzung eine erhöhte kapillare Durchlässigkeit nach sich zieht, könnte also die bakterielle Ausbreitung begünstigen, und außerdem die Bakterien durch einströmendes Plasma mit zusätzlichen Nährstoffen versorgen.

In der vorliegenden Arbeit wurde untersucht, ob ein Peptid (HKH20), dessen Sequenz aus der Bakterien- und Zell-bindenden Region in HK abgeleitet wurde, die Bindung und Aktivierung von Kontaktfaktoren an der bakteriellen Oberfläche verhindert. Weiterhin wurde untersucht ob sich HKH20 für die Behandlung einer experimentellen *S. pyogenes* Sepsis eignet.

Zunächst wurde - *in vitro* - nachgewiesen, dass HKH20 exklusiv den intrinsischen Gerinnungsweg inhibiert, sowohl im humanen als auch im murinen Plasma. Der extrinsische

Weg wird durch HKH20 nicht inhibiert. Das ist insofern bedeutend, als dass eine Inhibition des extrinsischen Weges auch immer mit einer Blutungsneigung einhergeht, was somit bei HKH20 ausgeschlossen werden konnte.

Weiterhin wurde *in vitro* nachgewiesen, dass HKH20 die Aktivierung von PK und Degradierung von HK sowohl an einer artifiziellen als auch an der bakteriellen Oberfläche verhindert.

In nachfolgenden Experimenten konnte dann gezeigt werden, dass eine Behandlung mit HKH20 in der mit *S. pyogenes* infizierten Maus eine Schädigung des Lungengewebes nahezu verhindern konnte. In den unbehandelten infizierten Mäusen waren die Lungen 18 h nach Injektion von *S. pyogenes* stark geschädigt, was durch Fibrin Ablagerungen, alveolare Schwellungen und Einblutungen gekennzeichnet war. In einer früheren Studie wurden solche Lungenschäden bei Patienten, die an einer fulminanten *S. pyogenes* Infektion verstorben waren, ebenfalls nachgewiesen (Ooe *et al*, 1999). Das in dieser Studie etablierte Sepsis-Mausmodell spiegelt also gut die Situation einer schweren *S. pyogenes* Infektion im Menschen wieder. Weiterhin deutet die im Mausmodell verlängerte aPTT auf eine systemische Kontaktaktivierung hin, was ebenso bei Patienten mit STSS nachgewiesen wurde (Sriskandan & Cohen, 2000).

Bereits in früheren Studien wurde gezeigt, dass die Blockierung des Kontaktsystems mit einem irreversiblen Kontaktsysteminhibitor schwere Lungenschäden bei einem Salmonellen-Sepsis Modell in der Ratte verhindert (Persson *et al*, 2000). Das dort verwendete Tripeptid Derivat (H-D-Pro-Phe-Arg-CMK) blockiert PK und FXII durch eine kovalente Bindung an die katalytische Domäne beider Proteasen (Ghebrehiwet *et al*, 1983; Tans *et al*, 1987). Trotzdem ist es ein unrealistischer Medikamentenkandidat, da die CMK-Gruppe (Chloromethylketon) toxisch ist, und das Peptid bei therapeutischer Dosis auch andere Proteasen inhibiert.

HKH20 hingegen bewirkt eine Kontaktsysteminhibition auf eine völlig andere Weise. Das Peptid verdrängt HK in seiner Bindung an artifizielle oder bakterielle Oberflächen und hat keinerlei Einfluss auf die enzymatische Funktion von FXII oder PK. Darüber hinaus haben wir gezeigt, dass HKH20 nicht toxisch ist, selbst bei einer Dosis die die therapeutische Dosis deutlich übersteigt. Es wurden weiterhin keine Anzeichen für Blutungen in den Organen der mit HKH20 behandelten Tiere gefunden, denn das Peptid blockiert ausschließlich das intrinsische System, welches keine Rolle für die physiologische Hämostase spielt. Dies und der Fakt, dass das Peptid schwere Lungenschäden verhinderte und bei einer kombinierten Behandlung mit Clindamycin sowohl die Überlebenszeit verlängerte als auch die Überlebensrate signifikant verbesserte, könnte ein neues therapeutisches Prinzip für schwere Infektionskrankheiten darstellen.

3.2 Die Deletion des L-Laktat-Dehydrogenase Gens in *S. pyogenes* ist mit einer verminderten SpeB- und Kontaktsystemaktivierung sowie einem hypovirulenten Phänotyp assoziiert (Anhang 2)

Eine Prävention der Kontaktaktivierung durch HKH20 hatte also einen positiven Effekt auf den Infektionsverlauf (siehe Anhang 1), und in einer weiteren Studie hatten wir *in vitro* gezeigt, dass Isolate von Patienten mit einer invasiven *S. pyogenes* Infektion das Kontaktsystem signifikant stärker aktivieren können als Isolate nichtinvasiver Infektionen (Nitzsche *et al*, 2015). Dies deutet darauf hin, dass eine Kontaktaktivierung - initiiert durch das Pathogen – mit einer erhöhten Virulenz assoziiert ist.

Im Rahmen dieser Studie (Anhang 2) haben wir den Einfluss einer Deletion der L-Lactat Dehydrogenase (LDH) in *S. pyogenes* untersucht, und sind dabei eher zufällig darauf gestoßen, dass diese Deletionsmutante in ihrer Fähigkeit das Kontaktsystem zu aktivieren beeinträchtigt ist. Weiterhin war die *ldh* Deletionsmutante in einem *Galleria mellonella* Infektionsmodell weniger virulent (Anhang 2).

Die LDH ist in Milchsäurebakterien für die Pyruvat-Degradierung und das Recycling von NAD^+ zuständig. Normalerweise betreibt *S. pyogenes* eine homofermentative Milchsäuregärung zur Energiegewinnung, und ist dabei auf die LDH angewiesen. Bei Verlust der LDH stellen die Bakterien ihren Stoffwechsel auf eine gemischte Säuregärung mit der Produktion von Ethanol, Azetat und Format um. Eine Deletion des *ldh*-Gens in *S. pyogenes* beeinträchtigte die Bakterien nicht in ihrem Wachstum auf komplexen oder chemisch definierten Labornährmedien (Fiedler *et al*, 2011). Allerdings war die *ldh* Deletionsmutante, im Gegensatz zum Wildtypen, nicht mehr in der Lage im Blut oder Plasma zu wachsen. Wir konnten nachweisen, dass die *ldh* Deletion zu einer verminderten Aktivierung der Kontaktfaktoren FXII/PK an der Oberfläche der Bakterien führte. HK band zwar nach wie vor an die Oberfläche der *ldh* Deletionsmutante, wurde dort aber nicht mehr degradiert. Eine Messung der SpeB Aktivität zeigte ebenfalls eine deutliche Reduktion, im Vergleich zum Wildtypen. Die reduzierte SpeB Aktivität war auf eine behinderte autokatalytische Aktivierung des Zymogens zurückzuführen, und ist höchstwahrscheinlich der Grund für die reduzierte Kontaktaktivierung in der *ldh* Deletionsmutante (Anhang 2).

Das von *S. pyogenes* sekretierte Protein SpeB (streptococcal pyrogenic exotoxin B) ist eine Cysteinprotease, welche als wichtiger Virulenzfaktor verschiedene humane und auch bakterielle Proteine degradiert. Dazu gehört, unter anderem, der Kofaktor des Kontaktsystems HK, wobei SpeB durch direkte Spaltung Bradykinin freisetzt (Herwald *et al*, 1996). SpeB scheint aber nicht nur HK direkt zu degradieren, sondern beeinflusst auch FXII und PK, denn

eine *speB*-Gen Deletionsmutante zeigte ebenfalls eine verminderte Aktivierung beider Faktoren an der Oberfläche der Bakterien (Anhang 2).

Zusammenfassend kann man sagen, dass die reduzierte Aktivität von Kontaktfaktoren in der Kombination mit dem Verlust der SpeB-Aktivität in der *ldh* Deletionsmutante zu einer reduzierten Virulenz beitrugen. Es bleibt die Frage warum die *ldh* Deletion in *S. pyogenes* die Aktivität von SpeB stört. Die Aufklärung dieses Mechanismus bedarf weiterer Untersuchungen.

3.3 Aktivierung des humanen Kontaktsystems durch neutrophil extracellular Traps (NETs) (Anhang 3)

Das humane Kontaktsystem interagiert mit der Oberfläche bakterieller und fungaler Pathogene. Bis heute allerdings ist ein endogener physiologischer Aktivator für FXII unbekannt, indessen wurden inzwischen verschiedene natürliche biologisch relevante Strukturen beschrieben, die zu einer Aktivierung des Systems führen wie z.B. Kollagen (van der Meijden *et al*, 2009), Polyphosphate (Smith *et al*, 2006) sowie fehlgefaltete Proteine und Proteinaggregate (Maas *et al*, 2008). In den folgenden beiden Studien (Anhang 3 und 4) haben wir gezeigt, dass bestimmte körpereigene Strukturen - freigesetzt von Zellen des angeborenen Immunsystems - Kontaktfaktoren binden und aktivieren.

Neutrophile Granulozyten sind ein wichtiger Teil der angeborenen Immunabwehr. Sie setzen neben antimikrobiellen Peptiden, Proteinasen und reaktiven Sauerstoffspezies auch sog. neutrophil extracellular Traps (NETs) zur Bekämpfung von Pathogenen frei (Papayannopoulos, 2017). Diese NETs bestehen hauptsächlich aus der DNA der Neutrophilen, den mit der DNA assoziierten Histon-Proteinen, sowie Proteinen der neutrophilen Granula wie neutrophiler Elastase oder Cathepsin G. Wenn die NETs in Kontakt mit Bakterien oder Pilzen kommen, dann verfangen diese sich in den netzartigen Strukturen, die aufgrund der enthaltenen neutrophilen Proteasen und Histone antimikrobiell wirken (Brinkmann *et al*, 2004).

Wenn man davon ausgeht, dass die Histon-Proteine aufgrund ihrer positiven Ladung an der negativ geladenen DNA „kleben“, dann ist es naheliegend anzunehmen, dass auch andere positiv geladene Proteine mit den NETs interagieren. Die Faktoren des Kontaktsystems werden an artifiziellen negativ geladenen Oberflächen aktiviert. Wir haben deshalb untersucht ob eine Aktivierung des Systems auch an NETs stattfindet. Wir konnten nachweisen, dass die Kontaktfaktoren HK und FXII sowohl an aufgereinigte DNA als auch an NETs binden. Dies führte *in vitro* zu einer Aktivierung von FXII und PK und der Freisetzung von Bradykinin (Anhang 3). Bereits 2007 wurde durch Kannemeier et. al gezeigt, dass extrazelluläre RNA

das Kontaktsystem aktiviert und thrombotisch wirkt (Kannemeier *et al*, 2007). Mit meiner Studie wurde erstmals gezeigt, dass auch extrazelluläre DNA in der Lage ist Kontaktfaktoren zu binden und zu aktivieren.

Die Freisetzung von NETs wird durch unterschiedliche Mechanismen initiiert, unter anderem auch durch Lipopolysaccharid, einem Membranbestandteil gramnegativer Bakterien (Brinkmann *et al*, 2004). In einer früheren Studie hatte man gezeigt, dass M-Protein, ein Oberflächenprotein und wichtiger Virulenzfaktor von *S. pyogenes* (siehe Review Oehmcke *et al*, 2010), mit Fibrinogen Komplexe bildet, welche eine Aktivierung von Neutrophilen induzieren, wodurch die Zellen ihre granulären Proteine freisetzen (Herwald *et al*, 2004). Wir haben deshalb weitergehend untersucht, ob diese Art der Aktivierung auch zu einer Freisetzung von NETs führt, und dies war der Fall (Anhang 3). Die Bildung von NETs scheint also dem Prinzip der Mustererkennung zu folgen und eine Aktivierung des Kontaktsystems an den NETs könnte die angeborene Immunantwort verstärken, indem proinflammatorisches Bradykinin freigesetzt wird.

3.4 Aktivierung des humanen Kontaktsystems durch prokoagulante Mikrovesikel (Anhang 4)

Das M-Protein aktiviert aber nicht nur Neutrophile, sondern auch Thrombozyten (Shannon *et al*, 2013) und Monozyten. Monozyten werden über den TLR2 Rezeptor aktiviert, was zu einer verstärkten Expression und Freisetzung der Zytokine IL-6, IL-1 beta und TNF alpha führt (Påhlman *et al*, 2006). Außerdem wird die Expression von Gerinnungsfaktor III (engl. auch tissue factor) und somit eine prokoagulante Aktivität der Monozyten über den extrinsischen Gerinnungsweg induziert (Påhlman *et al*, 2007). In einer meiner Studien haben wir weitergehend untersucht, ob die Aktivierung von Monozyten durch M-Protein zu einer Freisetzung von prokoagulanten Mikrovesikeln führt (Anhang 4).

Mikrovesikel sind kleine Vesikel, die aus der Membran nahezu aller Zellen kontinuierlich freigesetzt werden. In der älteren Literatur und auch in meiner Studie (Anhang 4) werden sie noch als Mikropartikel bezeichnet. Da es hier aber zu Verwechslungen mit nicht-biologischen Mikropartikeln kommen kann, und der Begriff „Partikel“ außerdem eher eine solide partikulare Struktur beschreibt, wird hier im Folgenden ausschließlich der Begriff Mikrovesikel (MV) verwendet. Dieser Begriff beschreibt eine biologische Membran-limitierte Struktur passender (György *et al*, 2011). In Abhängigkeit des Ausmaßes der Zellaktivierung unterscheiden sich MV in ihrer Zusammensetzung und Funktion. MV exponieren das negativ geladene Phospholipid Phosphatidylserin, welches in vielen biologischen Prozessen eine wichtige Rolle einnimmt. So ist es, exponiert auf der Oberfläche von Thrombozyten und MV, für die

Assemblierung von Gerinnungsfaktoren essentiell und verstärkt die prokoagulante Wirkung von Gerinnungsfaktor III (Butenas *et al*, 2009).

Im Rahmen dieser Studie haben wir gezeigt, das periphere monozytäre Blutzellen (PBMCs) nach Inkubation mit M-Protein und anderen bakteriellen Virulenzfaktoren prokoagulante MV freisetzen. Diese MV sind monozytären Ursprungs und tragen neben Gerinnungsfaktor III, Phosphatidylserin an ihrer Oberfläche. Im Rahmen dieser Studie haben wir erstmalig nachgewiesen, dass Phosphatidylserin eine wichtige Rolle bei der Bindung und Aktivierung der Kontaktfaktoren spielt. MV, die Phosphatidylserin exponieren, triggern die Gerinnung somit nicht nur über das extrinsische System (Gerinnungsfaktor III), sondern auch über das intrinsische System (Kontaktsystem). Die durch MV vermittelte Kontaktaktivierung trägt weiterhin zu einer Stabilisierung des Fibrin-Gerinnsels bei. Erstmalig konnten wir weiterhin zeigen, dass MV die Freisetzung von Bradykinin initiieren, womit diese MV nicht nur prokoagulante sondern auch proinflammatorische Wirkung besitzen (Anhang 4).

3.5 Eine neue Rolle von prokoagulanten MV in der frühen Wirtsantwort gegen *S. pyogenes* (Anhang 5)

Die Produktion von prokoagulanten MV steigt bei bestimmten Erkrankungen drastisch an. Hierzu zählen z.B. Thrombose, Kardiovaskulären Erkrankungen und Infektionskrankheiten (Nomura & Shimizu, 2015). Es stellte sich die Frage warum Immunzellen prokoagulante MV produzieren. Wir konnten sowohl in septischen Patienten (Trepesch *et al*, 2016) als auch in dem von mir etablierten Sepsis-Mausmodel (siehe Anhang 1) einen signifikanten Anstieg von prokoagulanten MV im Blut nachweisen (Anhang 5), was darauf hindeutet, dass ihre Freisetzung eine Immunantwort auf die invasive Infektion ist.

Durch *in vitro* Experimente haben wir gezeigt, dass prokoagulante MV an *S. pyogenes* binden und so die Generierung eines Fibrin-Koagels um die Bakterien induzieren können. Der durch MV induzierte Koagel verzögert eine bakterielle Ausbreitung und verfügt über antimikrobielle Eigenschaften. Dies konnten wir *in vivo* bestätigen, denn hier konnte eine lokale Behandlung mit prokoagulanten MV die Ausbreitung von *S. pyogenes* in die Organe der Maus eindämmen, was mit einer verbesserten Überlebensrate einherging. Die Bindung der MV an *S. pyogenes* erfolgt über Fibrinogen. Prokoagulante MV binden Fibrinogen mit einer hohen Affinität, was durch eine signifikant erhöhte Anzahl der Fibrinogen-bindenden Integrine CD18 und CD11b auf diesen MV bedingt sein kann (Anhang 5).

Wie wir in unserer Studie gezeigt haben, werden prokoagulante MV im frühen Stadium einer Infektion gebildet. Aufgrund ihrer lokalen Interaktion mit Bakterien schlussfolgern wir, dass die

Freisetzung dieser MV durch Immunzellen ein Teil der frühen angeborenen Immunantwort des Menschen darstellt.

Diese Hypothese wird durch eine Studie von Timar und Kollegen gestützt (Timár *et al*, 2013). Diese hatten kurz zuvor Daten veröffentlicht, die zeigen, dass neutrophile Granulozyten ohne jegliche Stimulation permanent MV produzieren, eine Beobachtung die auch wir mit PBMCs gemacht hatten (siehe Anhang 4). Nach Stimulation der Neutrophilen mit Bakterien erhöhte sich die Produktion der MV, welche nun aber veränderte biologische Eigenschaften aufwiesen. Die MV wirkten jetzt antibakteriell, und deshalb geht man davon aus, dass ihre Produktion ein weiterer extrazellulärer Mechanismus neutrophiler Granulozyten ist, um bakterielles Wachstum und Ausbreitung zu verhindern (Timár *et al*, 2013).

4. Zusammenfassung und Ausblick

Das humane Kontaktsystem aktiviert sowohl Blutgerinnung als auch Entzündung, zwei lebenswichtige Abwehrsysteme des Menschen. Wie ich im Rahmen meiner Studien erstmalig gezeigt habe, kann die Aktivierung des Systems durch die Bakterien selbst als auch durch Wirtsstrukturen, die als Antwort auf die Bakterien freigesetzt werden, erfolgen (siehe Abbildung 2). So erscheint die Rolle des Kontaktsystems während einer Infektion zwiespältig, denn einerseits unterstützt die lokale Aktivierung an der Oberfläche von Bakterien die lokale angeborene Immunantwort. Andererseits haben insbesondere pathogene Bakterien Mechanismen entwickelt Kontaktfaktoren zu aktivieren. Durch meine Studien konnte ich außerdem zeigen, dass dies mit einer erhöhten Virulenz einhergeht. Weiterhin hat eine systemische und unkontrollierte Kontaktaktivierung - initiiert z.B. durch eine massive Freisetzung von NETs oder prokoagulanten Mikrovesikeln - schädlichen Auswirkungen auf den Wirt.

Insbesondere bei einer Sepsis ist eine systemische Kontaktsystemaktivierung archetypisch, und es wurden viele unterschiedliche Tierstudien durchgeführt, in denen das System als therapeutisches Target fungierte. Hierbei wurden verschiedene Interventionen - welche FXII, PK oder Bradykinin-Rezeptoren während einer Sepsis inhibieren – getestet, und zeigten teilweise vorteilhafte Effekte für den Wirt (siehe Review Nicola, 2017).

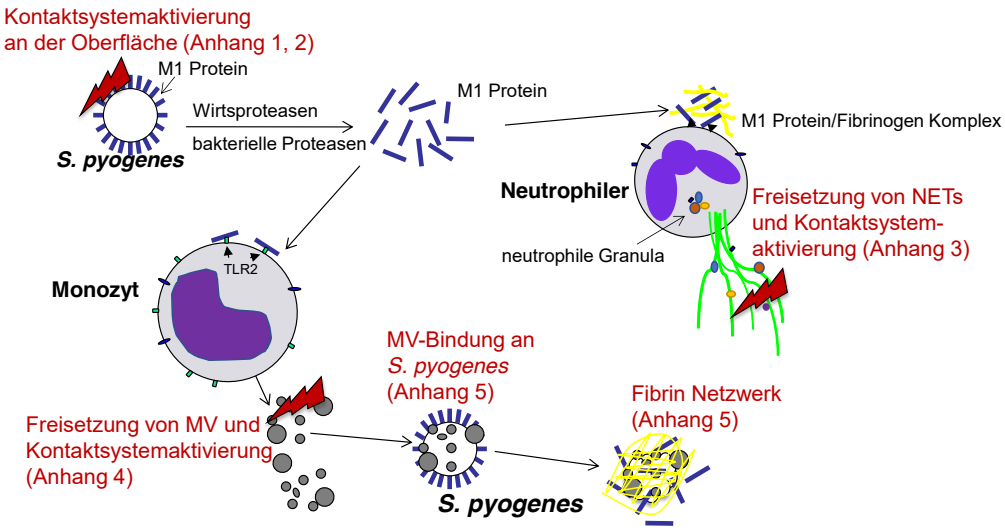

Abbildung 2: Mechanismen der Kontaktaktivierung während einer Infektion mit *S. pyogenes*. *S. pyogenes* bindet und aktiviert Kontaktfaktoren an seiner Oberfläche, was mit einer erhöhten Virulenz assoziiert ist (Anhang 1,2). Das bakterielle M-Protein kann sowohl durch Wirtsproteasen als auch durch bakterielle Proteasen freigesetzt werden. In löslicher Form aktiviert es Monozyten (Påhlman *et al*, 2006) als auch Neutrophile (Herwald *et al*, 2004). Die Aktivierung von Monozyten führt weiterhin zur Freisetzung von prokoagulanten MV (Anhang 4) welche auch das Kontaktsystem aktivieren können (Anhang 4). Im Rahmen einer lokalen Abwehrreaktion binden diese MV an *S. pyogenes* (Anhang 5) und induzieren damit ein Fibrinnetzwerk um die Bakterien, welches eine bakterielle Dissemination aufhält (Anhang 5). Neutrophile, welche durch Komplexe aus M-Protein und Fibrinogen aktiviert werden (Herwald *et al*, 2004), setzen NETs frei (Anhang 3). Die NETs binden und töten Bakterien ab (Brinkmann *et al*, 2004) und aktivieren das Kontaktsystem (Anhang 3).

Andererseits gibt es bis heute nur wenige Studien mit spezifischen Inhibitoren oder Knockout- bzw. Knockdown- Tieren, die aufzeigen würden, welche Rolle die einzelnen Kontaktfaktoren während einer mikrobiellen Sepsis genau spielen. Hier werde ich mit meiner Forschung zukünftig ansetzen.

Das Wissen über die molekularen Mechanismen, die in den Prozess der Kontaktaktivierung während einer Sepsis involviert sind, ist deshalb wichtig um neue therapeutische Strategien zu entwickeln. Diese sollten nicht nur darauf abzielen eine systemische Kontaktaktvierung zu verhindern, sondern auch dabei helfen Pathogene zu einem frühen Infektionszeitpunkt zu eliminieren.

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7. Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die Habilitationsschrift selbständig und ohne fremde Hilfe verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Rostock, den 25.07.2018

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Gemeinsame Drittmittelförderungen

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9. Vollständige Publikationsliste

Originalarbeiten: 19; Übersichtsartikel: 5; Hirsch-Faktor: 13

Originalarbeiten

2018	**IF**
Fiedler, T., Rabe, M., Mundkowski, R. G., **Oehmcke-Hecht, S.,** & Peters, K. (2018). Adipose-derived mesenchymal stem cells release microvesicles with procoagulant activity. *The International Journal of Biochemistry & Cell Biology*.	3.2
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Nitzsche, R., Köhler, J., Kreikemeyer, B. and **Oehmcke-Hecht, S**. (2016) *Streptococcus pyogenes* escapes killing from extracellular histones through plasminogen binding and activation by streptokinase. *J Innate Immun. 8(6)* Trepesch, C., Kreikemeyer, B., Glass, A., Schubert, J.K., and **Oehmcke-Hecht, S**. (2016). High intravascular tissue factor - but not pro-coagulant microvesicles - in septic patients is associated with a high SAPS II score. *J Intensive Care.* 4:34	3.8
2015	
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10. Vorträge auf Tagungen

2016
Veranstaltung: 3rd German Pneumococcal and Streptococcal Meeting,09.09.-10.09.16
Ort: Braunschweig, Deutschland
Präsentation: Streptococcus pyogenes escape killing from extracellular histones by plasminogen binding and streptokinase

2015:
Veranstaltung: Novel Concepts in Innate Immunity, 22.09.- 25.09.15
Ort: Tübingen, Deutschland
Präsentation: Novel Role for Pro-Coagulant Microvesicles in the Early Host Defense against Streptococcus pyogenes

Veranstaltung: 2nd German Pneumococcal and Streptococcal Meeting, 11.06.-13.06.15
Ort: Rostock, Deutschland
Präsentation: Contact system activation in *Streptococcus pyogenes* infections

2014
Veranstaltung: Gemeinsame Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) und der Vereinigung für allgemeine und angewandte Mikrobiologie (VAAM), 05.10.-08.10.14
Ort: Dresden, Deutschland
Präsentation: Novel Role for Pro-Coagulant Microvesicles in the Early Host Defense against *Streptococcus pyogenes*

2012
Veranstaltung: International Vascular Biology Meeting, 02.06.-05.06.12
Ort: Wiesbaden, Deutschland
Posterpräsentation (Kurzvortrag): Bacterial virulence factors trigger the release of pro-coagulant and proinflammatory microvesicles from blood mononuclear cells

2009
Veranstaltung: 1st International Symposium Infection of the Endothelium,27.08.-30.08.09
Ort: Dresden, Deutschland
Posterpräsentation (Kurzvortrag): Treatment of invasive *S. pyogenes* infection with a peptide derived from human high molecular weight kininogen

2008
Veranstaltung: XVI Lancefield International Symposium on Streptococci and Streptococcal Disease, 22.06.-26.06.08
Ort: Porto Heli, Griechenland
Präsentation: Inhibition of contact system activation at the bacterial surface of *S. pyogenes* prevents mice from lung lesions

Veranstaltung: 4th Swedish Natonal Meeting on Infection Biology, 13.11.-14.11.08
Ort: Malmö, Schweden
Präsentation: Treatment of invasive *S. pyogenes* infection with a peptide derived from high molecular weight kininogen

Anhang 1: *Treatment of invasive streptococcal infection with a peptide derived from human high-molecular-weight kininogen*

THROMBOSIS AND HEMOSTASIS

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

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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 resulting from 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 procoagulant 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. (Blood. 2009; 114:444-451)

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 and necrotizing fasciitis.[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 proinflammatory cytokines.[2]

Previous work has shown that *S 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 is composed of 4 components: factor XI (FXI), FXII, plasma kallikrein (PK), and high-molecular-weight kininogen (HK). Under physiologic 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). On activation, the contact system triggers the intrinsic pathway of coagulation via activation of FXI by FXII and evokes the release of bradykinin (BK) from the HK precursor by the action of PK. BK, a peptide consisting of 9 amino acids, is a potent proinflammatory mediator. Thus, BK has been shown to evoke the generation of nitric oxide and other inflammatory substances (eg, 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. Although the local activation of the contact system is considered to have a beneficial effect for the human host, that is, 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.

Methods

Materials

The *S pyogenes* strain AP1 (40/58) of serotype M1 was from the World Health Organization (WHO) Collaborating Center for Reference and

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Research on Streptococci (Prague, Czech Republic). Bacteria were grown in Todd-Hewitt broth (TH; Difco) at 37°C in the presence of 5% CO_2. Fresh frozen plasma from healthy persons was obtained from the blood bank at Lund University Hospital (Lund, Sweden) and kept frozen at −80°C until use.

The synthetic peptides HKH20 (HKHGHGHGKHKNKGKKNGKH) and GCP28 (GCPRDIPTNSPELEETLTHTITKLNAEN) 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 minute followed by the addition of 100 μL aPTT reagent (aPTT Automate; Diagnostica Stago) for 60 seconds at 37°C. Clotting was initiated by the addition of 100 μL of a 25-mM $CaCl_2$ 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

Fifty-milliliter overnight cultures of *S pyogenes* AP1 bacteria were washed 3 times with 50 mM Tris-HCl (pH 7.5), resuspended, and diluted to a final concentration of 2×10^{10} colony-forming units (CFU)/mL in 50 mM Tris-HCl/50 μM $ZnCl_2$ buffer. A total of 200 μL of bacteria was incubated with 60 μL of HKH20 or GCP28 (final concentration 50 or 100 μM) for 30 seconds before the addition of 200 μL human plasma. Samples were incubated for 30 minutes at 37°C with shaking. After centrifugation, pellets were washed 2 times in 50 mM Tris (pH 7.5) centrifuged, resuspended in 100 μL 50 mM Tris-HCl/50 μM $ZnCl_2$ buffer containing 1 mM of the chromogenic substrate S-2302, and incubated for 30 minutes 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 ~ 0.4 at 620 nm) in TH broth, washed, and diluted in 50 mM Tris-HCl (pH 7.5); 50 μL of bacteria (2×10^6 CFU/mL) was incubated together with HKH20 at various concentrations for 1 hour 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 3 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 μM $ZnCl_2$ buffer. A total of 100 μL of bacteria was incubated with 30 μL of HKH20 or GCP28 (final concentration, 50 or 100 μM) for 30 seconds before the addition of 100 μL human plasma. Samples were incubated on a rotator at room temperature for 15 minutes unless indicated otherwise. After incubation, the bacteria were washed 2 times in 50 mM Tris (pH 7.5), centrifuged, and resuspended in 50 μL of 50 mM Tris-HCl/50 μM $ZnCl_2$ buffer. The suspensions were allowed to stay at room temperature for 15 minutes, followed by centrifugation at 10 000*g*. Supernatants were collected and kept at −20°C until Western blot analysis.

Electrophoresis and Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Neville.[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) for 15 minutes, served as controls. Before loading, samples were boiled in sample buffer containing 2% (wt/vol) SDS and 5% (vol/vol) beta-mercaptoethanol for 10 minutes. 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 (PBS)–Tween containing 5% (wt/vol) nonfat dry milk at room temperature for 30 minutes, washed 3 times with PBS-Tween for 5 minutes, and incubated with sheep antibodies against HK and its degradation products (1:3000 in the blocking buffer) at room temperature for 60 minutes. 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 minutes. Bound secondary antibodies were detected by the chemiluminescence method.[18]

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 to 12 weeks old, were injected intraperitoneally with 100 μL of the bacterial solution, or with 100 μL of bacteria together with 100 μL HKH20 (2 mg/mL, which corresponds to 200 μg/mouse) mixed directly before injection, or with 200 μL PBS alone (control group). Alternatively, mice were treated with 50 μg aprotinin/mouse (Merck, > 5 TIU/mg protein). After 18 hours, mice were killed 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 to 12 hours after infection. To measure clotting times, 8 groups of female BALB/c mice were infected; and after various time points (0, 4, 6, 10, 12, 18, 24, and 42 hours), 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 one-tenth volume of 3.8% trisodium citrate. Plasma was separated by centrifugation, and clotting times were measured as described earlier. To determine bacterial dissemination, spleens were removed and bacterial counts were determined.

PMN depletion was induced by intraperitoneal injection of the anti–mouse Ly-6G (Gr-1) antibody (eBioscience; 100 μg/mouse) 8 hours before infection. Neutropenia was confirmed before starting the infection by manual white blood cell count and fluorescence-activated cell sorter (FACS) analysis.[19] To investigate leukocyte recruitment into the peritoneal cavity, mice were killed, 5 mL PBS was injected, and after massage of the peritoneum the fluid was removed and analyzed. Statistical analysis was performed using GraphPad Prism, Version 4.00. The *P* value was determined using the unpaired *t* test (comparison of 2 groups) or the log-rank test (comparison of survival curves). All animal experiments were approved by the regional ethical committee for animal experimentation, the Malmö/Lund djurförsöksetiska nämnd, Lund District Court, Lund, Sweden (permit M209-06).

Pharmacokinetics of HKH20

HKH20 was labeled with the fluorescent dye AlexaFluor 555 (Invitrogen) according to the manufacturer's protocol. The labeled peptide or the same amount of dye (control) was injected intraperitoneally into mice. Animals were killed after 15, 30, 60, and 120 minutes. Blood samples were collected and organs (lung, spleen, liver, and kidney) removed. The fluorescence of the blood samples was measured with a Multilabel counter (PerkinElmer Life and Analytical Sciences). Tissue samples were fixed at 4°C for 24 hours in buffered 4% formalin (pH 7.4; Kebo), dehydrated, and imbedded in paraffin (Histolab Products), 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 histopathologic evaluation

Mice were killed, and lungs rapidly removed and fixed at 4°C for 24 hours in buffered 4% formalin (pH 7.4; Kebo). Tissues were dehydrated and

imbedded in paraffin (Histolab Products), cut into 4-μm sections, and mounted. After removal of the paraffin, tissues were stained with Mayers hematoxylin (Histolab Products) and eosin (Surgipath Medical Industries). Mouse organs were fixed in 4% paraformaldehyde (in PBS, pH 7.4) and processed for routine histopathologic evaluation. Slides were viewed with a Nikon ECLIPSE 80i microscope using a Plan APO 20×/0.75 DIC N2 lens. Images were acquired using NIs-Elements Imaging Software (Version 2.33).

Scanning electron microscopy

Lung samples from the mice were fixed as previously described.[20] After fixation, samples were washed, dehydrated, critical point dried, and sputtered with palladium/gold as described earlier.[21] Specimens were examined in a Jeol J-330 scanning electron microscope (JEOL) operated at an acceleration voltage of 5 kV, working distance of 10 minutes and a magnification of 300× to 2500×. Images were captured with a Satan Multiscan 791 CCD camera (Satan Inc).

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 (eg, 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 sulfate.[25] Our findings that radiolabeled HKH20 binds directly to kaolin and *S pyogenes* (data not shown) are 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 FXII prevents activation of the contact system.[26] 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 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 TCT, measuring thrombin-induced fibrin-network formation, were not affected in human plasma (Figure 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 preincubated with HKH20, GCP28, or buffer alone, followed by incubation with human plasma. After 15 minutes, 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).

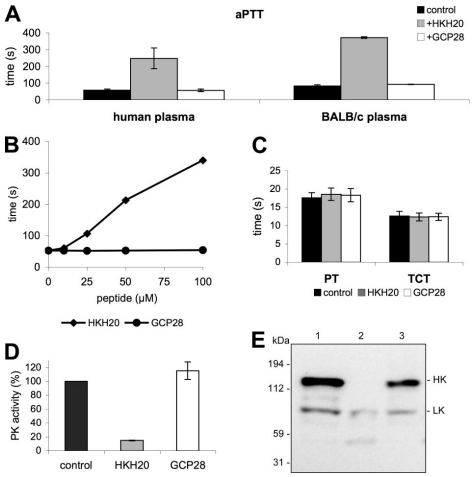

Figure 1. HKH20 interferes with the intrinsic pathway of coagulation. (A) Human plasma or BALB/c mouse plasma was incubated with 50 μM HKH20, GCP28, or buffer alone (control) for 60 seconds and analyzed by the aPTT test. (B) Human plasma was incubated with increasing amounts of HKH20 or GCP28, and the aPTT was measured. (C) Normal human plasma was incubated with 50 μM HKH20, GCP28, or buffer alone (control) for 60 seconds and analyzed by the PT and the TCT tests. (D) Human plasma was incubated with kaolin in the presence of 50 μM HKH20, GCP28, or buffer alone (control) for 15 minutes. Plasma was removed by centrifugation and pelleted kaolin was washed and resuspended in substrate buffer. After 15 minutes of incubation, plasma kallikrein activity was measured in a substrate assay. Data are presented as percentage activity compared with the control; values are mean ± SD (n = 3). (E) Human plasma was incubated with buffer (lane 1), kaolin (lane 2), or kaolin and 50 μM HKH20 (lane 3) for 15 minutes. Samples were analyzed by Western blotting with antibodies identifying HK and LK.

As expected, HKH20 efficiently blocks PK activity, whereas the control peptide GCP28 has no influence on the enzymatic activity (Figure 1D). Because HK is a substrate for activated PK, Western blot analysis was used to test whether inhibition of PK activity prevents HK degradation. Kaolin was preincubated with HKH20 and then mixed with human plasma for 15 minutes. 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 HK,[27] and a polyclonal antiserum against HK will also react with LK. Figure 1E depicts intact HK at 120 kDa (Figure 1E lane 1) and processed HK after kaolin treatment (Figure 1E lane 2), which triggers the conversion of HK from a single chain to a 2-chain protein.[22] When plasma was incubated with kaolin in the presence of HKH20, cleavage of HK was blocked and intact HK was detected (Figure 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 minutes, 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

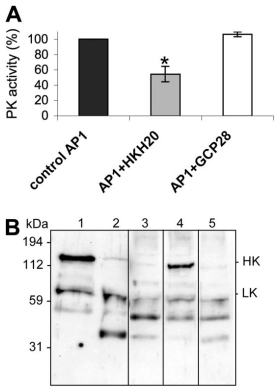

Figure 2. HKH20 inhibits *S pyogenes*-induced contact activation. (A) Human plasma was incubated with *S pyogenes* bacteria in the presence of 50 μM HKH20, GCP28, or buffer alone (control) for 30 minutes. Plasma was removed by centrifugation, and bacteria were washed and resuspended in substrate buffer. After 30 minutes of incubation, plasma kallikrein activity was measured in a substrate assay. Data are presented as percentage activity compared with the control; values are mean plus or minus SD (n = 3). *$P < .05$ by *t* test. (B) Human plasma was incubated with *S pyogenes* bacteria in the presence or absence of HKH20 or GCP28 for 15 minutes. Bacteria were washed, resuspended in buffer, incubated for 15 minutes, and spun down. Supernatants and plasma (nontreated or kaolin-treated) were analyzed by SDS-PAGE and Western blotting with antibodies against HK and LK: lane 1, normal plasma; lane 2, kaolin-treated plasma; lane 3, plasma proteins absorbed and released by *S pyogenes*; lane 4, plasma proteins absorbed and released by *S pyogenes* in the presence of 100 μM HKH20; lane 5, plasma proteins absorbed and released by *S pyogenes* in the presence of 100 μM GCP28. Vertical lines have been inserted to indicate a repositioned gel lane.

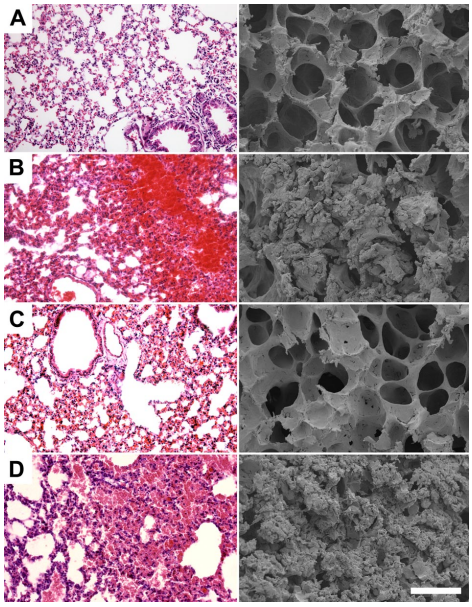

Figure 3. HKH20 prevents lung damage in BALB/c mice infected intraperitoneally with *S pyogenes*. Light microscopy (left) and scanning electron microscopy (right) of representative mouse lung tissue sections are shown. Mice were injected intraperitoneally with (A) 200 μL PBS, (B) 5×10^6 CFU *S pyogenes*, (C) 5×10^6 CFU *S pyogenes* and 200 μg HKH20, and (D) 5×10^6 CFU *S pyogenes* and 275 μg GCP28. Bars represent 250 μm (light microscopy) and 50 μm (scanning electron microscopy).

treatment with HKH20 evoked a significant decrease in PK activity compared with controls incubated with buffer alone or with the GCP28 peptide (Figure 2A). Next we analyzed whether the coapplication of HKH20 prevents HK processing under these experimental conditions. The bacteria were preincubated with HKH20 for 1 minute, followed by the addition of plasma for 15 minutes. They were then washed and resuspended in buffer, followed by an additional incubation step for 15 minutes 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 (Figure 2B). Nontreated and kaolin-treated plasma served as negative and positive controls, respectively (Figure 2B lanes 1 and 2). Western blot analysis of plasma proteins bound to and released from the streptococcal surface revealed that HK was degraded (Figure 2B lane 3). However, when bacteria were preincubated with 100 μM HKH20 before plasma was added, the degradation of HK bound to the bacteria was decreased (Figure 2B lane 4). As a control, *S pyogenes* was treated with peptide GCP28, which did not prevent HK cleavage (Figure 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 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 killed and examined. Histopathologic 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 seconds; PT, 11.2 ± 0.5 seconds) as those from the PBS-treated mice (aPTT, 35.6 ± 4.5 seconds; PT, 11.5 ± 0.2 seconds).

To test the effect of HKH20 in a sepsis model, mice were injected intraperitoneally with 5×10^6 CFU *S pyogenes*, and different groups (n = 5/group) were treated with peptides HKH20, GCP28, or with PBS. Mice injected intraperitoneally 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 killed and lungs were examined by light and scanning electron microscopy. Figure 3 shows representative micrographs of lungs from noninfected mice, displaying no indication of pulmonary damage (Figure 3A), whereas mice infected with *S pyogenes* alone (Figure 3B) or together with peptide GCP28 (Figure 3D) had severe hemorrhage, alveolar swelling, and fibrin deposits. Such lung lesions were almost completely prevented when bacteria were injected together with HKH20

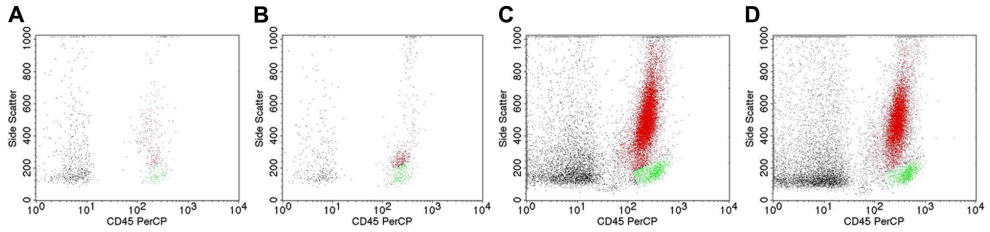

Figure 4. Leukocyte recruitment is not impaired by HKH20. FACS analysis of peritoneal lavage from noninfected mice injected with PBS (A) or HKH20 (B), mice injected intraperitoneally with 5×10^6 CFU *S pyogenes* in the absence (C) or presence (D) of 200 μg HKH20. Peritoneal lavage was analyzed 18 hours after infection. All leukocytes are CD45 positive, and neutrophil populations (red) are separated from monocyte populations (green) based on their side scatter pattern.

(Figure 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 PMN recruitment[25] and to be antibacterial.[29] To test whether any of these properties contributes to the protective effect of the peptide, we first investigated the effect of HKH20 on neutrophil influx. Mice were infected intraperitoneally with the bacteria in the presence or absence of HKH20. Eighteen hours after infection, the animals were killed 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 leukocytes, which was not significantly changed when mice were treated with HKH20 ($14.5 \times 10^5 \pm 3.2 \times 10^5$ leukocytes/mL in the untreated group vs $14.7 \times 10^5 \pm 2.7 \times 10^5$ leukocytes/mL in the HKH20-treated group). Injection of HKH20 in uninfected mice did not cause a significant influx of leukocytes (Figure 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 approximately 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 intraperitoneally, 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 compared with 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 killed, and lungs and spleens were 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 (supplemental Figure 1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). 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 (Figure 5). Comparing normal infected and neutropenic infected mice in the absence of treatment, we found that lung lesions were reduced from 85% to 61%, respectively (Figure 5A-B), demonstrating that PMN activation and recruitment contribute to, but are not the main cause of, the damage. However, a significantly reduced damage was observed when infected mice were treated with HKH20 (Figure 5C-D) or aprotinin (Figure 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 because (1) there was no significant difference in the bacterial load of the spleens from normal and neutropenic animals, regardless of HKH20 treatment, and (2) the concentration of HKH20 used in these experiments was too low to be antibacterial (supplemental Figure 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 (AlexaFluor 555) was injected intraperitoneally into noninfected

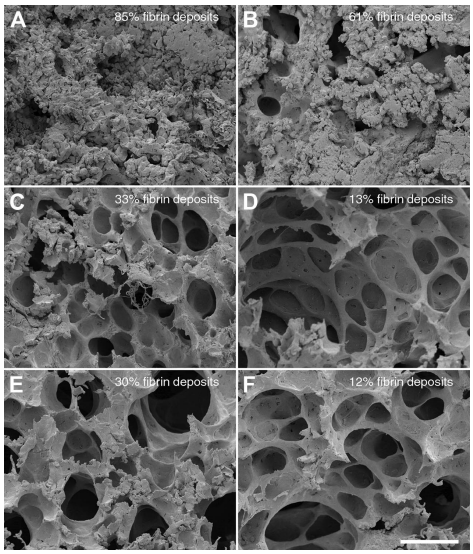

Figure 5. Comparison of lung lesions in normal and neutropenic mice infected with *S pyogenes*. Scanning electron micrographs of representative mouse lung tissue sections are shown. Normal (A,C,E) or neutropenic (B,D,F) mice were injected intraperitoneally with (A-B) 5×10^6 CFU *S pyogenes*, (C-D) 5×10^6 CFU *S pyogenes*, and 200 μg HKH20 (E-F) 5×10^6 CFU *S pyogenes* and 50 μg aprotinin. Bar represents 50 μm.

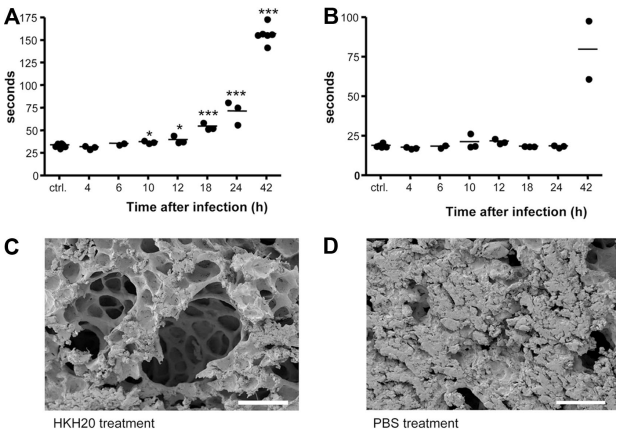

Figure 6. Contact activation in vivo and treatment with HKH20. Mice were injected subcutaneously in the neck with 2×10^7 CFU *S pyogenes* bacteria, plasma was collected at 0, 4, 6, 10, 12, 18, 24, and 42 hours after infection (n = 2-6/group), and aPTT (A) and PT (B) were measured immediately. *$P < .05$; ***$P < .001$. (C) Scanning electron microscopy of representative mouse lung tissue sections is shown. Mice were injected subcutaneously with 2×10^7 CFU *S pyogenes* and treated intraperitoneally 8 hours after infection with 200 μg HKH20 (C) or 100 μL PBS (D). Lungs were taken 18 hours after infection. Bar represents 100 μm.

mice (200 μg/animal). Animals were killed after 15, 30, 60, and 120 minutes 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 minutes after injection, whereas after 120 minutes most of the signal was gone (supplemental Figure 2). Microscopic analysis of the organs revealed an accumulation of the peptide in lung, spleen, and liver after 15 minutes, which then decreased within 2 hours after injection of the peptide, whereas it appears to accumulate in the kidney within this time (supplemental Figure 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 in the scruff of the neck[31] at a dose of *S pyogenes* causing more than 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 and 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 PT was not prolonged during the first 24 hours of infection but also increased after 42 hours (Figure 6B). Based on these results and considering the clearance rate of HKH20 in plasma, we decided to administer HKH20 8 hours after infection. Electron microscopic analysis of lung biopsies from these animals revealed a significant reduction of lung lesions (Figure 6C) compared with infected mice treated with the buffer control (Figure 6D). In line with these findings, it should be mentioned that infected mice exhibited a time-dependent increase in PK activity (up to 24 hours), 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 ($P = .017$). 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 ($P = .054$) 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

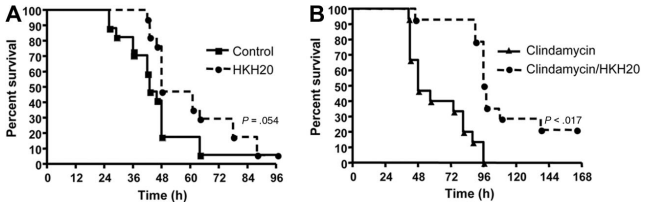

Figure 7. HKH20 improves survival in a mouse model of *S pyogenes* sepsis. (A) Mice were injected subcutaneously in the neck with 2×10^7 CFU *S pyogenes* bacteria and treated with 100 μL HKH20 (200 μg; □) or 100 μL PBS (■) intraperitoneally 8 hours after infection (n = 4 or 5/group). Mortality was recorded for a period of 5 days. The experiment was repeated 4 times, and the results from a total of 17 animals per group are shown. (B) Mice were infected subcutaneously with 2×10^7 CFU *S pyogenes* bacteria and treated with 10 mg/kg clindamycin (▲) or 200 μg HKH20 and 10 mg/kg clindamycin (□) in a volume of 200 μL PBS. Treatment was intraperitoneal 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 3 times, and the results from a total of 15 animals per group are shown.

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 because clindamycin is a recommended treatment for patients with invasive streptococcal infection. Initial experiments showed that *S pyogenes* strain AP1 is clindamycin sensitive (minimum inhibitory concentration < 0.064 mg/L, E test). Moreover, when bacteria were grown in plasma together with 10 mg/L clindamycin ($156 \times$ minimum inhibitory concentration) 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 (Figure 6A). This time point was then also chosen for the first intraperitoneal treatment with clindamycin (10 mg/kg) and HKH20 (200 μg/mouse). Both substances were subsequently administered intraperitoneally at 42, 48, and 72 hours after infection. In the control group, mice were injected intraperitoneally with clindamycin (10 mg/kg) only (Figure 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 ($P < .001$). 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 with 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 because of the anticoagulative 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 past 40 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 pathologic 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 et al that patients with hypotensive septicemia have significantly decreased levels of contact factors,[32] whereas in 1992 Pixley et al reported that low levels of FXII and HK in patients with systemic inflammatory response syndrome correlate with a fatal outcome of the disease.[2] 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.[34]

It is estimated that invasive *S pyogenes* infections cause more than 150 000 deaths annually worldwide,[35] and the prognosis is especially poor in cases with lung hemorrhage.[36-38] Histologic examination of lungs recovered from patients who died of fulminant *S pyogenes* infection revealed severe intra-alveolar hemorrhage,[38] and in these patients the aPTT was dramatically prolonged.[38] 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 because lung lesions decreased from 85% to 61%, respectively, in neutropenic mice (Figure 5A-B). This is in concordance with previous studies showing that M1 protein, a classic virulence determent 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 blocked.[31] However, in that study, the inhibitor was given before the infection became invasive (30 minutes) and the mice were killed after 6 hours, 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 hours after subcutaneous 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 tripeptide derivative (H-D-Pro-Phe-Arg-CMK) forms a covalent link with the catalytic pocket of PK and FXII.[41,42] Because 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, which 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

Contribution: S.O. performed research, analyzed the data, and wrote the paper; O.S., A.L., and A.I.O. performed research; M.v.K.-B. contributed analytic tools; M.M. contributed analytic tools and performed research; and L.B. and H.H. designed research and wrote the paper.

Conflict-of-interest disclosure: Hansa Medical (Lund, Sweden), which in part supported this study, has filed patent applications on HKH20. S.O., M.M., L.B., and H.H. are listed as inventors, and the applications are pending. The remaining authors declare no competing financial interests.

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Anhang 2: *Deletion of the L-Lactate Dehydrogenase Gene ldh in Streptococcus pyogenes Leads to a Loss of SpeB Activity and a Hypovirulent Phenotype*

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Deletion of the L-Lactate Dehydrogenase Gene *ldh* in *Streptococcus pyogenes* Leads to a Loss of SpeB Activity and a Hypovirulent Phenotype

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Streptococcus pyogenes uses lactic acid fermentation for the generation of ATP. Here, we analyzed the impact of a deletion of the L-lactate dehydrogenase gene *ldh* on the virulence of *S. pyogenes* M49. While the *ldh* deletion does not cause a general growth deficiency in laboratory media, the growth in human blood and plasma is significantly hampered. The *ldh* deletion strain is furthermore less virulent in a *Galleria mellonella* infection model. We show that the *ldh* deletion leads to a decrease in the activity of the cysteine protease SpeB, an important secreted virulence factor of *S. pyogenes*. The reduced SpeB activity is caused by a hampered autocatalytic activation of the SpeB zymogen into the mature SpeB. The missing SpeB activity furthermore leads to increased plasmin activation and a reduced activation of the contact system on the surface of *S. pyogenes*. All these effects can be reversed when *ldh* is reintroduced into the mutant via a plasmid. The results demonstrate a previously unappreciated role for LDH in modulation of SpeB maturation.

Keywords: SpeB, LDH, *Galleria mellonella*, *Streptococcus pyogenes*, contact system

INTRODUCTION

Streptococcus pyogenes (group A streptococcus, GAS) is an important human pathogen. It is equipped with a large number of virulence factors. The expression of these virulence factors is tightly controlled by a complex network of regulatory proteins and sRNAs (Fiedler et al., 2010; Patenge et al., 2013; Walker et al., 2014). Taxonomically, GAS belongs to the Lactobacillales meaning that it converts carbohydrates to lactic acid. In lactic acid bacteria, the major enzyme responsible for pyruvate degradation and recycling of the NAD^+ reduced during glycolysis is L-lactate dehydrogenase (Fiedler et al., 2011; Levering et al., 2012, 2016; Feldman-Salit et al., 2013). In a previous study, we could show that deletion of the L-lactate dehydrogenase gene in GAS M49 strain 591 does not affect the growth of the bacteria in complex or chemically defined laboratory media. The bacteria are simply switching their metabolism from homofermentative lactate production to the mixed acid branch with production of ethanol, acetate, formate, and an additional ATP per glucose (Fiedler et al., 2011). Here, we show that the deletion of the *ldh* gene in GAS M49 strain 591 leads to a significant loss of fitness in human blood or plasma, a decreased

contact system activation on the bacterial surface, an increased plasmin/streptokinase (Ska) activity and decreased virulence in a *Galleria mellonella* infection model. We show that this phenotype can be explained by the loss of activity of the streptococcal cysteine protease SpeB in the *ldh* deletion strain.

The GAS protein originally named streptococcal pyrogenic exotoxin B (SpeB) is neither pyrogenic nor is it an exotoxin. Instead, it is a potent secreted cysteine protease and an important virulence factor in GAS (Nelson et al., 2011). The *speB* gene is transcribed as a bicistronic mRNA with the *spi* gene encoding the SpeB inhibitor protein Spi (Kagawa et al., 2005). Intracellularly, Spi probably prevents SpeB from cleaving cytoplasmic GAS proteins (Kagawa et al., 2005). Extracellularly, SpeB is activated from its 40 kDa zymogen into an active 28 kDa enzyme by autocatalytic cleavage under reducing conditions (Doran et al., 1999). The mechanisms triggering the activation process are not fully understood. There is experimental evidence that cell wall-anchored M protein is involved in the activation of SpeB into the mature enzyme (Collin and Olsén, 2000). SpeB has been shown to cleave immunoglobulins, complement factors, and numerous host matrix and plasma proteins (Kapur et al., 1993a,b; Herwald et al., 1996; Collin and Olsén, 2001; Terao et al., 2008; Honda-Ogawa et al., 2013). Among the plasma proteins degraded by SpeB is high molecular weight kininogen (HK), a component of the human contact system (Herwald et al., 1996). The contact system, also known as the intrinsic pathway of coagulation, consists of four proteins, factor XI, factor XII (FXII), plasma kallikrein (PK) and HK (Frick et al., 2006). FXII is activated on negatively charged surfaces. Activated FXII activates (i) factor XIa which triggers the intrinsic pathway of coagulation, and (ii) prekallikrein into PK which cleaves HK into kinins, e.g., the proinflammatory bradykinin, and smaller peptides, e.g., the antimicrobially active NAT26 (Frick et al., 2006). Prekallikrein can also be activated by the plasma protease plasmin. The activation of plasminogen into plasmin is usually mediated by human tissue or urokinase plasmin activators tPA and uPA, but can also be activated via Ska, another secreted GAS virulence factor (Nitzsche et al., 2015). SpeB potently degrades Ska, thereby reducing plasmin activity (Svensson et al., 2002). Furthermore, bacteria can directly interact with different contact system components (Nickel and Renne, 2012). GAS can bind HK via the M protein and cleave it via SpeB (Ben Nasr et al., 1997). Hence, there is a complex network of interactions of GAS with coagulation factors and, consequently, with hemostasis in the human host.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *S. pyogenes* serotype M49 wild type (WT) strain 591 was obtained from R. Lütticken (Aachen). The L-lactate production deficient mutant (Δ*ldh*) of *S. pyogenes* M49 has been described previously (Fiedler et al., 2011). Generally, bacteria were grown in Todd Hewitt broth (Oxoid) supplemented with 0.5% yeast extract (THY; Oxoid) at 37°C in a 5% CO_2/20% O_2 atmosphere.

Genetic Manipulations

For the construction of a complementation plasmid a fragment comprising the *ldh* gene and its native promoter (280 bp upstream of the start codon) of the *S. pyogenes* M49 591 WT strain was amplified by PCR using a Phusion High Fidelity DNA polymerase. This fragment was inserted into the shuttle vector pAT19 (Trieu-Cuot et al., 1991) via *Bam*HI and *Sal*I restriction sites. Correct insertion of the fragment was confirmed by sequencing. The resulting plasmid was transferred into the *S. pyogenes* M49 591 *ldh* deletion strain via electroporation. The resulting strain *S. pyogenes* M49 591 Δldh::ldh was tested for LDH activity to confirm functional expression of the plasmid-located *ldh* gene.

Measurement of LDH Activity

For measurements of LDH activity, *S. pyogenes* strains were grown in THY under standard conditions. At mid-exponential growth phase (OD at 600 nm = 0.5) cells of 2 ml culture were pelleted by centrifugation and washed twice in sodium phosphate buffer (50 mM, pH 6.8). Intracellular proteins were released by enzymatic degradation of the cell wall with phage lysin PlyC for 10 min at 37°C as described previously (Nelson et al., 2006; Koller et al., 2008). Subsequently, lysates were centrifuged for 10 min at 20,000 *g* and 50 µl of the supernatants were applied in the activity assay either directly or in a 1:25 dilution in sodium phosphate buffer. LDH activity was determined via measurement of the conversion of NADH to NAD^+ as described previously (Levering et al., 2012). In brief, the reaction mixture contained 0.5 mM fructose-1,6-bisphosphate, 0.17 mM NADH, 10 mM sodium pyruvate and 50 µl sample in 50 mM sodium phosphate buffer (pH 6.8) in a total volume of 1 ml and the absorption at 340 nm was measured for 5 min. The specific activity is given in units per milligram protein, where 1 unit is defined as conversion of 1 µM NADH per minute. Protein concentrations in the *S. pyogenes* lysates were measured with the Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific).

Proteome Analysis by NanoLC-HDMS^E

In-solution digestion of proteins with trypsin in sodium deoxycholate-containing buffer was performed as previously described (Masuda et al., 2008; Pade et al., 2017) using total protein extracts prepared in a Precellys 24 homogenizer (peqLab Biotechnologie GmbH, Erlangen, Germany). Mass spectrometry was performed on a Synapt G2-S mass spectrometer (Waters, Manchester, United Kingdom) coupled to a nanoAcquity UPLC system (Waters) as described (Pade et al., 2017). In short, peptides mixtures were separated on an analytical column (ACQUITY UPLC HSS T3, 1.8 µm, 75 µm × 250 mm, Waters) at a flow rate of 300 nl/min using a gradient from 3 to 32% acetonitrile in 0.1% formic acid over 150 min. The SYNAPT G2-S instrument was operated in data-independent mode (Geromanos et al., 2009), characterized by parallel fragmentation of multiple precursor ions in combination with ion-mobility separation as an additional dimension of separation (referred to as HDMS^E) (Shliaha et al., 2013; Distler et al., 2014). Samples were measured in duplicate.

NanoLC-HDMS^E Data Processing, Protein Identification, and Quantification

Progenesis QI for Proteomics version 2.0 (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom) was used for raw data processing, protein identification, and label free quantification. For the database search a database containing 1701 protein sequences from *S. pyogenes* serotype M49 (strain NZ131) (UniProt release 2016_05) appended with the sequences of rabbit phosphorylase B (P00489) and porcine trypsin was compiled. One missing cleavage site was allowed, oxidation of methionine residues was considered as variable modification, and carbamidomethylation of cysteines as fixed modification. The false discovery rate based on the search of a reversed database was set to 1%. Peptides were required to be identified by at least three fragment ions and proteins by at least seven fragment ions and two peptides. With the subsequent filtering steps peptides were removed that had (i) a peptide score below 5.96, (ii) a mass error above 10 ppm, (iii) less than six amino acid residues. Proteins were quantified by the absolute quantification Hi3 method using Hi3 rabbit phosphorylase B Standard (Waters) as reference (Silva et al., 2006). Protein abundance changes by a factor of at least two, accompanied by ANOVA p-values <0.05 for the comparison between the respective groups were regarded as significant.

Survival Assays

The survival assays in human blood or plasma were performed as described previously (Nakata et al., 2009). In short, bacteria grown in THY medium were harvested in the exponential growth phase (optical density at 600 nm of 0.4–0.5). Bacteria were suspended in phosphate-buffered saline (PBS), the optical density at 600 nm was adjusted to 0.3 and this suspension was diluted 1:10,000 in PBS. The viable counts were determined by plating serial dilutions on THY agar plates. Twenty microliters of the bacterial suspension were inoculated into 480 µl of fresh citrated human blood or plasma and incubated for 3 h at 37°C with rotation. The viable counts were determined by plating serial dilutions on THY agar plates and related to the inoculum.

Ethics Approval Statement

The protocol for the collection of human blood for the blood and plasma survival assays was approved by the Ethikkommission an der Medizinischen Fakultät der Universität Rostock (ethics committee vote: A 2014-0131). The experiments were conducted in accordance with the ICH-GCP guidelines. Informed consent was obtained from all subjects.

Western Blot Analysis

For sampling, overnight cultures were set to 2×10^8 CFU/ml and 250 µl were mixed with equal amount of human normal plasma and incubated at 37°C for 60 min with shaking (600 rpm). Incubation of plasma with PBS, bacteria with PBS and plasma with kaolin served as controls. Cells were pelleted by centrifugation, washed three times in PBS, suspended in 100 µl glycine (0.1 M) and incubated at room temperature for another 10 min. Cells were pelleted by centrifugation and supernatants were neutralized by the addition of 20 µl Tris–HCl (1 M, pH 8.4). A total of 100 µl of the suspensions were mixed with 20 µl SDS sample buffer (5×). SDS-PAGE was performed as described earlier (Neville, 1971). Following SDS-PAGE, separated proteins were transferred onto nitrocellulose membranes. Western blot analyses were performed with sheep antibodies against HK (1:3,000; Affinity Biologicals) and its degradation products as described previously (Mattsson et al., 2001). Chemiluminescence was detected on a Kodak ID3.5 Image Station 440CF.

Dot Blot Analysis

For dot blot analysis, 100 µl of filter-sterilized (0.2 µm) supernatants (either pure or diluted with fresh THY) of overnight cultures of the bacteria in THY medium were transferred on a nitrocellulose membrane using a Bio-Dot® microfiltration apparatus (Bio-Rad). The detection was performed with a SpeB (bD-12) antibody from goat (1:1,000) and a donkey-anti-goat IgG-HRP secondary antibody (1:1,000; both Santa Cruz Biotechnology) as described elsewhere (Mattsson et al., 2001).

Measurement of FXII/PK Activity

FXII/PK activity on bacterial surfaces exposed to plasma was measured using chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-pNA·2HCl; Chromogenix). Ten-milliliter overnight cultures (THB) of the *S. pyogenes* M49 strains were washed three times with 50 mM Tris–HCl (pH 7.5) and diluted (final concentration, 3×10^7 CFU/ml) in 50 mM Tris buffer. Then, 100 µl of bacterial suspension was mixed with 100 µl of plasma or buffer (control), followed by incubation at 37°C for 30 min. After centrifugation, the pellets were washed three times and suspended in 200 µl of buffer containing 1 mM substrate S-2302. After 60 min at 37°C, the samples were centrifuged, and the absorbance of the supernatants was measured at 405 nm in a microplate reader. Control values (bacteria incubated in buffer) were used as a blank. No endogenous proteolytic activity was measured when S-2303 was incubated with bacteria in the absence of plasma.

Measurement of Plasmin Activity

To measure the plasmin activity on bacterial surfaces exposed to plasma, bacteria were grown overnight in 10 ml of THB, washed three times with PBS, and diluted (final concentration 5×10^7 CFU/ml) in PBS. Next, 200-µl bacterial suspensions were mixed with 200 µl of plasma or buffer, followed by incubation for 3 h. After three further washing steps with PBS, the pellet was suspended in Tris–NaCl buffer (19.2 mM/1.062 M; pH 7.5) containing 20 µg/ml of the chromogenic substrate S-2251 (H-D-Val-Leu-Lys-pNA·2HCl; Sigma), followed by an incubation for 60 min at 37°C. Samples were centrifuged, and the absorbance of the supernatants was measured at 405 nm in a microplate reader.

Measurement of SpeB Activity

For determination of SpeB activity in bacterial culture supernatants bacteria were grown for 8 h in THY (Becton, Dickinson and Company), diluted 1:100 in fresh THY medium and further cultivated for 12 h. Cells were pelleted, 1 ml per supernatant was filter-sterilized (0.2 µm pore size), and 50 µl of sterile supernatants were activated with 2 mM

dithiothreitol (DTT) for 30 min at 37°C. Then, 150 µl of substrate solution (1 mM N-benzoyl-Pro-Phe-Arg-p-NA, 60 mM sodium phosphate pH 6) were added and incubated for 2 h at 37°C. Absorbance was measured immediately after substrate addition and after the 2 h incubation at 405 nm in a microplate reader. As a specificity-control, samples without DTT activation and samples with addition of 0.05 mM cysteine protease inhibitor E64 (Sigma) were measured simultaneously.

Galleria mellonella Infection Model

Larvae of the greater wax moth *G. mellonella* were obtained from Reptilienkosmos (Niederkrüchten, Germany). For infection experiments, *S. pyogenes* strains were grown overnight in THY, washed twice in a 0.75% NaCl solution and suspended in 0.75% NaCl to a final concentration of 1.5–2 × 10^8 CFU/ml. Larvae with a weight of 150–200 mg were inoculated with 10 µl of this bacterial suspension, resulting in an infection dose of 1.5–2 × 10^6 CFU/larva. Bacteria were injected into the hemocoel of the larvae between the last pair of legs using a microapplicator (World Precisions Instruments, Sarasota, United States) and a fine dosage syringe (Omnican®-F, 0.01–1 ml, 0.30 × 12 mm, B. Braun AG, Melsungen, Germany). As a control, larvae were mock inoculated with 10 µl of a 0.75% NaCl solution. Survival of the larvae was observed for seven days. Larvae were regarded dead when they were not moving upon repeated physical stimulation with tweezers (Mukherjee et al., 2010).

RESULTS

As previously reported, a deletion of the *ldh* gene in *S. pyogenes* M49 strain 591 leads to a metabolic switch from homofermentative lactate production to mixed acid fermentation but not to general growth retardation of the bacteria in laboratory media (Fiedler et al., 2011). Here we aimed to elucidate whether the loss of L-lactate dehydrogenase has an impact on the fitness of *S. pyogenes* in infection-related conditions. For that purpose we first constructed a complementation strain carrying a pAT19-based plasmid containing the *S. pyogenes* M49 strain 591 *ldh* gene with its native promoter. Protein extracts obtained from cells in the exponential growth phase (OD = 0.5) in THY medium showed the same specific LDH activity for complementation strain and WT, while extracts of the *ldh* deletion strain were completely devoid of L-LDH activity (**Figure 1A**). Furthermore, the abundance of proteins was analyzed in total extracts from exponentially growing bacteria of all three strains by label-free protein quantification using ion mobility-enhanced data-independent acquisition ($HDMS^E$) (Distler et al., 2016). Protein extracts from three biological replicates per strain were pooled for these measurements. In the WT and complementation strain, pyruvate branching enzymes were at similar levels after overnight growth in THY, with relatively high abundance of lactate dehydrogenase and relatively low levels of pyruvate formate lyase and alcohol dehydrogenase as compared to the *ldh* deletion strain (**Figure 1B**). Abundance of glycolytic enzymes, i.e., phosphofructokinase, fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, enolase, and pyruvate kinase, was similar in all three strains (**Figure 1B**). Hence, in terms of pyruvate branching, the complementation strain is able to restore the WT phenotype. The abundance of the enzymes encoded upstream and downstream of LDH (NADH oxidase and gyrase A) was unaffected in the *ldh* deletion strain (Supplementary Table 1).

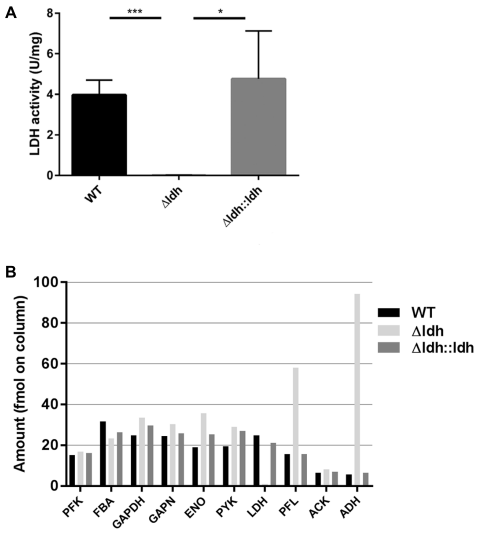

FIGURE 1 | LDH-activity **(A)** and abundance of glycolytic and pyruvate branching enzymes **(B)**. LDH activity was measured in protein extracts of cultures grown to the exponential growth phase (OD_{600} = 0.5) in THY ($n = 3$, $^*p < 0.05$, $^{***}p < 0.001$, unpaired two-tailed t-test). For the measurement of protein abundances **(B)**, protein extracts of three biological replicates of THY cultures (OD_{600} = 0.5) were pooled and subjected to label-free protein quantification using nanoLC-$HDMS^E$.

Next, we assessed the ability of WT, *ldh* deletion mutant and complementation strains to multiply in citrated human blood and plasma, respectively. As shown in **Figure 2A**, the *ldh* deletion strain had significantly lower multiplication rates in blood than WT and complementation strain. The phenotype in plasma resembled that in blood (**Figure 2B**). This indicates that not only cellular components but also soluble components such as plasma proteins seem to be responsible for the reduced multiplication of the *ldh* deletion strain.

Since the contact system is one of the humoral innate immune mechanisms that is activated by GAS M49 (Nitzsche et al., 2015), we analyzed the extent of contact activation at the surface of the bacteria. For that purpose the activity of the proteases FXII and PK bound to the bacterial surface after incubation in plasma was determined in a colorimetric assay. As depicted in **Figure 3A**, the FXII/PK activity at the surface of the *ldh* deletion strain was significantly reduced compared to the WT. As contact system

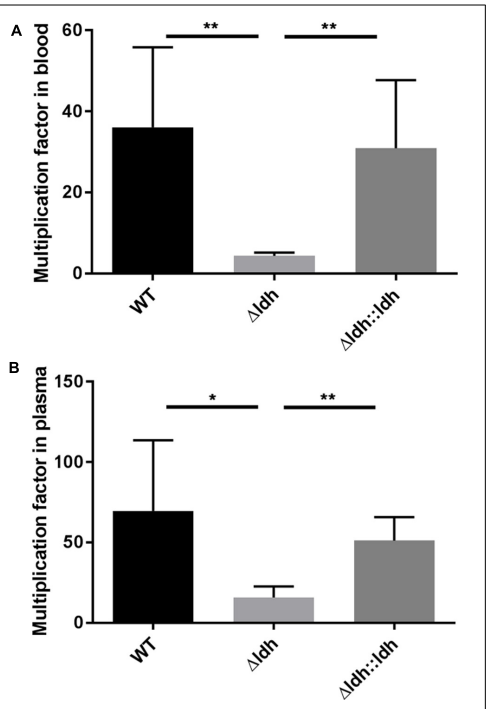

FIGURE 2 | Growth in human blood **(A)** and plasma **(B)**. Bacteria were incubated in citrated blood or plasma for 3 h. Viable counts before and after the incubation were used to calculate multiplication rates ($n \geq 5$, $^{*}p < 0.05$, $^{**}p < 0.01$, unpaired two-tailed *t*-test).

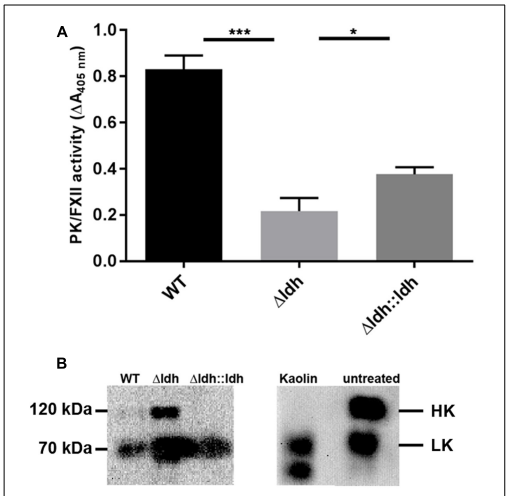

FIGURE 3 | PK/FXII activity **(A)** and Western blot analysis of HK bound at the bacterial surface **(B)**. PK/FXII activity was determined in plasma samples exposed to the bacteria and depicted as rates of conversion of a chromogenic plasma kallikrein substrate ($n = 3$, $^{*}p < 0.05$, $^{***}p < 0.001$, unpaired two-tailed *t*-test). For Western blots, plasma proteins eluted for bacteria or kaolin (as a positive control) were applied to SDS-PAGE, blotted on nitrocellulose membranes, and detected with a kininogen-specific primary antibody and a HRP-conjugated secondary antibody.

activation is accompanied with a degradation of HK, we analyzed whether intact HK is bound at the surface of M49, M49$_{\Delta ldh}$, and M49$_{\Delta ldh::ldh}$ strains. For that purpose, bacteria were incubated in plasma, and, after washing, the adsorbed proteins were eluted from the surface and analyzed by western blot with antibodies directed against HK and low-molecular weight kininogen (LK). LK is a shorter splice variant of HK (Furuto-Kato et al., 1985), but has no function in contact activation (Lalmanach et al., 2010), and the polyclonal antiserum against HK also reacts with LK. Plasma alone or plasma treated with kaolin (a contact activator) served as negative and positive control, respectively. As depicted in **Figure 3B**, in untreated plasma HK could be detected at 120 kDa and LK at 66 kDa. In the kaolin-treated plasma, HK was processed and consequently the signal at 120 kDa disappeared. A similar pattern was obtained in the eluate samples from the bacteria, which contain plasma proteins adsorbed at the surface of *S. pyogenes* and its mutants (**Figure 3B**). The 120 kDa HK signal was completely absent in eluate samples from the WT and the complemented strain (**Figure 3B**). This is in contrast to the *ldh* deletion strain, where intact 120 kDa HK was eluted from the bacterial surface (**Figure 3B**). This implies that the *ldh* deletion strain does not degrade surface-bound HK, which is in line with its lower surface FXII/PK activity as described above (**Figure 3A**).

A major streptococcal protein interacting with the contact system is the secreted cysteine protease SpeB (Nelson et al., 2011) as it can degrade HK into kinins (Herwald et al., 1996). On the other hand SpeB also degrades bacterial virulence factors such as Ska and M protein (Walker et al., 2014). Therefore, we determined SpeB activity in the supernatants of THY cultures (**Figure 4A**). In the supernatant of the *ldh* deletion strain no SpeB activity was detectable. In contrast, in the supernatants of WT and complementation strain significant SpeB activity was measured (**Figure 4A**). Both—PK and SpeB—efficiently cleave HK (Herwald et al., 1996), thus the completely restored SpeB activity in the complemented strain probably induces the complete HK degradation, although FXII/PK levels are not fully restored in the complemented strain (**Figure 3B**). In line with the absence of SpeB activity, the *ldh* deletion strain showed a significantly higher surface plasmin activity after incubation in human plasma compared to WT and complementation strain (**Figure 4B**). The loss of SpeB activity in the *ldh* deletion strain is apparently not due to reduced SpeB production, as in dot blot analysis with SpeB-specific antibodies SpeB could be detected in the supernatants of all three strains (**Figure 4C**). The reason for the absence of SpeB activity in the culture supernatants of the *ldh* deletion strain rather seems to be a disturbed autocatalytic

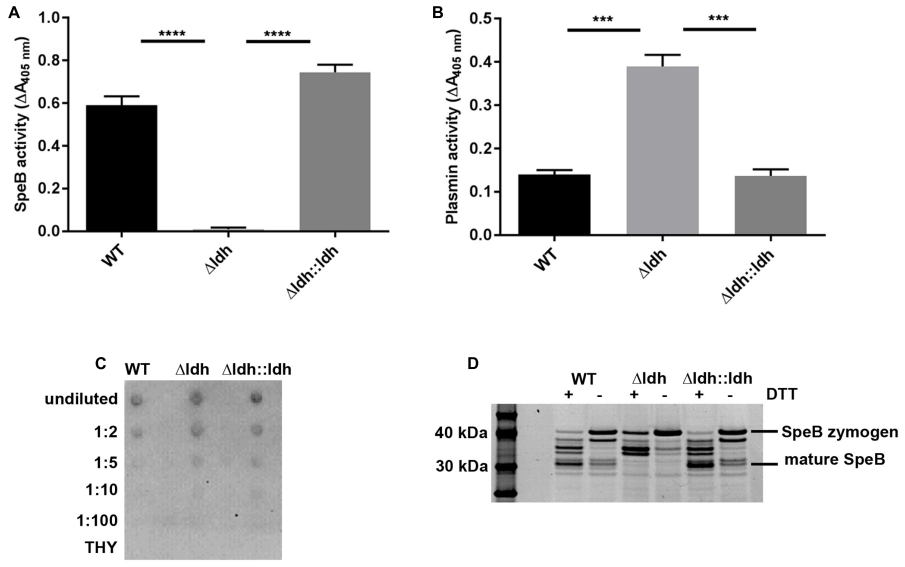

FIGURE 4 | The *ldh* deletion has an impact on SpeB and plasmin activity. SpeB activity **(A)** was determined in culture supernatants of overnight cultures in THY ($n = 4$, $^{****}p < 0.0001$, unpaired two-tailed *t*-test), plasmin activity **(B)** was measured on the surface of bacteria after incubation in pooled human plasma ($n = 3$, $^{***}p < 0.001$, unpaired two-tailed *t*-test), dot blot analysis of SpeB abundance in culture supernatants with SpeB specific antibodies **(C)**, non-reducing SDS-PAGE showing SpeB activation via DTT treatment **(D)**.

activation of the 40 kDa SpeB zymogen into the 28 kDa mature SpeB. In the culture supernatant of the *ldh* deletion mutant there was only a faint signal for the mature 28 kDa SpeB detectable. Instead, intermediates frequently occurring in SpeB maturation accumulated (Doran et al., 1999; Chen et al., 2003) (**Figure 4D**). In contrast, in supernatants of WT and complementation strain the mature SpeB was much more abundant than in the *ldh* deletion strain (**Figure 4D**).

Sequencing of the *speB* locus in all three strains revealed no mutations in the promoter region or the coding sequence of the *speB* gene. In time-course experiments with exposure of the culture supernatants of all three strains to DTT for up to 7 h, we observed that in WT and complementation strain, the SpeB zymogen is almost completely converted into the mature SpeB after 1 h (**Figure 5** and Supplementary Figure 1). In contrast, a visible accumulation of mature SpeB in the culture supernatants of the *ldh* deletion strain could only be observed after 3–4 h incubation under reducing conditions (**Figure 5** and Supplementary Figure 1). The addition of 30 mM L-lactate to the culture supernatant of the mutant prior to the DTT activation did not lead to faster maturation of SpeB, indicating that missing lactate in the supernatants is not responsible for the delay in SpeB activation (see Supplementary Figure 2).

The streptococcal endopeptidase HtrA (aka DegP) has previously been reported to support the maturation of SpeB (Lyon and Caparon, 2004; Rosch and Caparon, 2005).

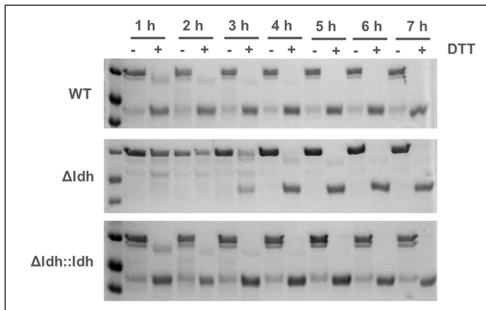

FIGURE 5 | Impact of the *ldh* deletion on SpeB maturation. SpeB maturation in the culture supernatants of wild type, *ldh* deletion and complementation strain in the presence of DTT over 7 h was analyzed via SDS-PAGE.

Furthermore, cell-wall anchored M protein has been described to be necessary for maturation of SpeB in a M1 serotype GAS strain (Collin and Olsén, 2000). In our proteomic data, we, however, found HtrA abundances unchanged in the *ldh* deletion strain compared to WT and complementation strain (see Supplementary Table 1). The M protein amount in WT and *ldh* deletion strain was similar as well, while in the complementation

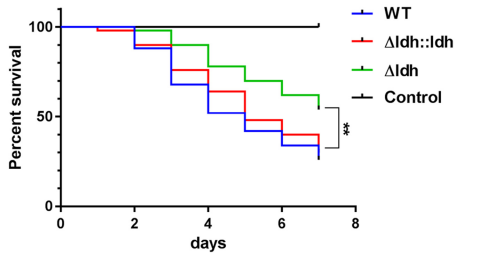

FIGURE 6 | *Galleria mellonella* infection model. Survival of *G. mellonella* larvae after infection with WT, *ldh* deletion and complementation strains ($n = 50$, $^{**}p < 0.01$, log-rank test).

strain a moderately increased M protein amount was measured (see Supplementary Table 1).

To elucidate the impact of the *ldh* deletion on virulence of the M49 strain 591 *in vivo*, larvae of the greater wax moth *G. mellonella* were used as model organisms. *G. mellonella* is an easy-to-handle and well-established invertebrate infection model organism that reliably reflects differences in virulence of *S. pyogenes* as they can also be observed in more complex mammalian model organisms (Olsen et al., 2011; Cook and McArthur, 2013; Loh et al., 2013). Here, the larvae were inoculated with 1.5–2 $\times$ 10^6 CFU/animal by injecting the bacterial suspension into the hemocoel of the larvae. As controls, larvae were mock-inoculated with an equal volume of a sterile physiological NaCl solution. Survival of the larvae was followed for 7 days after infection. Larvae infected with the *ldh* deletion mutant survived the 7-day period in a significantly higher proportion (54% living animals at day 7) than larvae infected with the WT (26%) or complementation strain (30%) (**Figure 6**). Hence, the reduced virulence of the *ldh* deletion strain *in vitro* is also displayed *in vivo* in the *Galleria* infection model.

DISCUSSION

The secreted streptococcal cysteine protease SpeB is one of the central virulence factors of GAS. SpeB is associated with an invasive phenotype of the bacteria (Carroll and Musser, 2011). SpeB degrades host proteins as well as proteins secreted by GAS (Nelson et al., 2011). After biosynthesis, cytosolic SpeB is probably kept inactive by the SpeB inhibitor protein Spi, which is encoded on a bicistronic operon with the *speB* gene (Kagawa et al., 2005). During secretion of SpeB via the ExPortal protein secretion microdomain located Sec translocon of *S. pyogenes* (Rosch and Caparon, 2004, 2005), the leader peptide is cleaved off, and the protein is released into the extracellular space as a 40 kDa zymogen (Doran et al., 1999). The SpeB zymogen can either be autocatalytically cleaved into the mature enzyme or be cleaved by the mature enzyme itself. Over the maturation process, up to eight intermediate forms of SpeB can be found (Doran et al., 1999; Chen et al., 2003).

Here, we show that in an *ldh* deletion strain of *S. pyogenes* M49 strain 591, the activation of the SpeB zymogen into the mature active SpeB enzyme is hampered. Full virulence and a complete regain of SpeB activity is achieved by extrachromosomal expression of the *ldh* gene in the *ldh* deletion background. The missing SpeB activity likely contributes to the reduced virulence of the M49 *ldh* deletion strain. In accordance with our findings, it has previously been shown that SpeB promotes survival of *S. pyogenes* in human blood (Terao et al., 2008) and that the absence of SpeB renders *S. pyogenes* more prone to phagocytosis by neutrophils (Lukomski et al., 1998). In accordance with that, GAS M49 mutants lacking active SpeB have a decreased virulence in murine intraperitoneal and skin infection models (Lukomski et al., 1997, 1998, 1999; Meinert Niclasen et al., 2011).

Next to the impact on susceptibility toward phagocytosis, another virulence mechanism associated with SpeB is the ability to cleave HK, with the release of pro-inflammatory kinins, independent from PK activation (Herwald et al., 1996). The data of the present study show that upon *ldh* deletion the bacteria have a reduced PK/FXII activity and reduced HK cleavage on their surface. Both—PK and SpeB—efficiently cleave HK, resulting in release of BK, which leads to increased vascular permeability promoting spread of the infection and providing nutrients to growing bacteria (Ben Nasr et al., 1997). Thus reduced PK/FXII activity in combination with the lack of SpeB activity reduces HK cleavage and may further contribute to a reduced virulence.

In addition to the decreased virulence due to the loss of SpeB activity, the missing ability of lactate fermentation might represent a metabolic disadvantage in blood or the larvae. For *Streptococcus pneumoniae* an *ldh* deletion strain has been described to be avirulent in an intravenous mouse infection model (Gaspar et al., 2014). The authors attribute the loss in virulence to a decreased fitness of the *S. pneumoniae ldh* deletion strain, since the bacteria are forced to use the inefficient mixed acid branch of pyruvate metabolism in the absence of lactate dehydrogenase. This is also reflected by a general growth deficiency of the *S. pneumoniae ldh* deletion strain in laboratory media (Gaspar et al., 2014). In *S. pyogenes* the *ldh* deletion also leads to a shift from homofermentative lactate production to mixed acid fermentation (**Figure 1B**) (Fiedler et al., 2011). The *ldh* deletion, however, had only marginal effects on growth efficiency in laboratory media, a finding that also holds for other homofermentative lactic acid bacteria, i.e., *Enterococcus faecalis* and *Lactococcus lactis* (Fiedler et al., 2011). Therefore, a general growth deficiency of the *S. pyogenes ldh* deletion strain cannot be held responsible for the loss in virulence of this strain. Anyway, it still cannot be excluded that the *ldh* deletion represents a disadvantage in terms of metabolic fitness in infection relevant conditions.

The main question remaining is how the *ldh* deletion interferes with SpeB maturation. Hampered SpeB activation has also been reported by Cho and Kang (2013) for a c-di-AMP phosphodiesterase mutant of *S. pyogenes* HSC5, a M14 strain. As in the study of Cho and Kang (2013), the underlying mechanism of interference with SpeB maturation remains obscure and needs further investigation.

AUTHOR CONTRIBUTIONS

SO-H, BK, and TF planned the experiments. SM, JW, and LN did the experiments. SO-H, TF, and SM analyzed the data. TF wrote the manuscript. SO-H, SM, and BK edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01841/full#supplementary-material

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Anhang 3: *Activation of the Human Contact System on Neutrophil Extracellular Traps*

Short Communication

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Activation of the Human Contact System on Neutrophil Extracellular Traps

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Key Words
Contact system · DNA-protein interaction · Immunity · Innate · Polymorphonuclear leukocytes · Streptococcus

Abstract
Pattern recognition is an integral part of the innate immune system. The human contact system has been shown to interact with the surface of many bacterial and fungal pathogens, and once activated leads to the generation of antimicrobial peptides and the proinflammatory mediator bradykinin. Here we show that apart from these surfaces also neutrophil extracellular traps (NETs) provide a surface that allows the binding and activation of the contact system. In addition, we present evidence that M1 protein, a streptococcal surface protein, in concert with human fibrinogen triggers polymorphonuclear neutrophils to form NETs.

Introduction

Apart from antimicrobial peptides, leukocyte-borne proteinase, and reactive oxidative species, polymorphonuclear neutrophils (PMNs) may also release their nuclear content to form web-like structures upon activation. These neutrophil extracellular traps (NETs) consist of DNA and granule-derived proteins such as neutrophil elastase, cathepsin G, bactericidal/permeability-increasing protein and antimicrobial peptides [1, 2]. As such, NETs are capable to assemble and kill various bacterial and fungal species and, thus, they are now considered as an important part of the innate immune system [1]. Assuming that most neutrophil-released proteins stick to NETs because of their positive net charge, it is tempting to speculate that also other positively charged proteins, derived for instance from plasma, interact with NETs. Notably, activation of the contact system requires the assembly of its factors on a negatively charged artificial or biological surface [3–6]. The contact system comprises 3 serine proteinases factor XI (FXI), factor XII (FXII), plasma kallikrein (PK) and 1 nonenzymatic cofactor high molecular weight kininogen (HK), which is in complex with either PK or FXI [for a review, see 7]. Upon activation, 3 events occur: (1) activation of the intrinsic pathway of coagulation via activation of FXI by activated FXII, (2) release of bradykinin (BK) from the HK precursor by the action of activated PK and (3) the generation of antimicrobial peptides [for a review, see 7].

Streptococcus pyogenes is a major human pathogen that mainly causes superficial and self-limiting skin and throat infections, which can occasionally develop into serious and life-threatening conditions such as streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis [for a review, see 8]. It was recently published that M1 protein, one of the classical virulence determinants of *S. pyogenes*, forms complexes with fibrinogen which then

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are able to activate PMNs [9]. In the present study we show that this activation also evokes very efficiently the formation of NETs. We further show that purified DNA as well as NET-DNA absorbs purified HK and FXII, and when added to plasma activates the contact system.

Methods

PMN Purification and Activation

PMNs were purified from blood of healthy donors using Polymorphprep (Fresenius) as described earlier [9]. The cells were washed and diluted in ME-Medium (Invitrogen) with HEPES buffer. Fifty microliters of 3×10^6 cells/ml were seeded on poly-lysine-treated slides and activated with 100 mU/ml glucose oxidase (GO; Sigma) or 100 nM IL-8 (Pepro Tech) or 5 μl of preformed M1 protein/fibrinogen complexes for 60 min (20 μg purified M1 protein [10] added to 6 mg fibrinogen; the precipitate was suspended in 100 μl MEM).

DNA Preparation

DNA from purified PMNs was isolated using the DNazol Reagent (Invitrogen) as described by the manufacturer.

Measurement of PK Activity

PK activity in human plasma was measured after activation with purified DNA as follows. Ten microgram per milliliter DNA together with the chromogenic substrate (S2302) was incubated with human plasma for 60 min at 37°C. The absorbance was then measured after different time points at 405 nm. As a control, DNA were degraded with 200 mU DNase I (Ambion) for 30 min at 37°C before adding to plasma. To measure PK activity on activated PMNs, 200 μl PMNs in MEM (3×10^6 cells/ml) were stimulated with GO or IL-8 as described above. DNase I (200 mU; Ambion) was added to the controls and incubation was continued for 30 min at 37°C. The supernatants were gently removed and the cells were incubated with human plasma from the same donor for another 15 min at 37°C. After removing the plasma, cells were incubated in physiological salted sodium citrate buffer (13 mM) containing the chromogenic substrate (S2023). Absorbance was measured over a time period of 60 min at 37°.

BK Measurements

Different concentrations of purified DNA (1, 10 and 100 μg/ml) were incubated with human plasma for 15 min at 37°C, and the BK content was measured as described earlier [11]. As controls, DNA samples were degraded with DNase I prior to incubation with plasma.

Statistical Analysis

Statistical analysis was performed using GraphPadPrism 4.00. The p value was determined by using the unpaired t test (comparison of 2 groups).

Scanning Electron Microscopy

Activated PMNs were immobilized on poly-lysine cover slides, before and after treatment with DNase I, fixed in 2.5% (v/v) glutaraldehyde and prepared for scanning electron microscopy as described earlier [9].

Transmission Electron Microscopy

PMNs were activated as described above, and incubated with gold-labeled HK and FXII for 30 min at room temperature. Cells were then fixed with 2.5% glutaraldehyde, dehydrated and embedded in Epon as described earlier [9]. For negative staining, purified DNA (20μg/ml) were mixed with 20 nM HK and 20 nM FXII for 20 min at room temperature. Samples were then processed as previously reported [12].

Results and Discussion

Activation of the Contact System by Soluble DNA Purified from PMNs

In the present study we wished to investigate whether NETs can provide a surface that allows the assembly and activation of contact system. As the skeletal structure of NETs is primarily built up by PMN-derived DNA [1], in the first set of experiments we focused on soluble DNA purified from PMNs (also see Materials and Methods) and tested its interaction with the contact factors HK and FXII. To this end, purified human HK and FXII were incubated with DNA, negative stained, and examined with a transmission electron microscope. Figure 1a shows negative-stained DNA, HK and FXII. When all components were mixed together, the micrographs revealed that DNA avidly recruited HK and FXII, which, interestingly, were always found in proximity to each other. To test whether binding of HK and FXII to soluble DNA is followed by an activation of the contact system, we monitored PK hydrolysis in DNA-treated plasma. As shown in figure 1b, an increase in the cleavage of a PK-specific substrate was seen when soluble DNA was added to plasma, but not with DNase I-treated DNA. It should be noted that the DNase treatment had no influence on the activity of PK in kaolin-activated plasma, implying that hydrolysis of the PK substrate is not influenced by DNase or contaminants thereof (data not shown). Finally, we measured whether activation of PK is followed by the release of BK, which was indeed seen when DNA was mixed with plasma and prevented when DNA was degraded by DNase I prior adding to plasma (fig. 1c). Taken together, the data demonstrate that purified DNA has the ability to assemble and activate the contact system.

The Contact System Is Bound and Activated on NETs

To test whether not only soluble DNA but also NETs can activate the contact system, PMNs were stimulated with glucose oxidase or IL-8. Both substances have been earlier reported to trigger NET formation [13] and this was also confirmed in our studies by fluorescence and

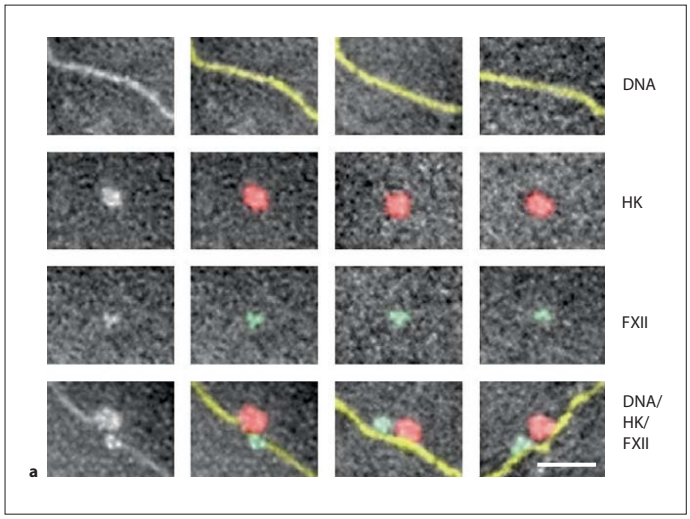
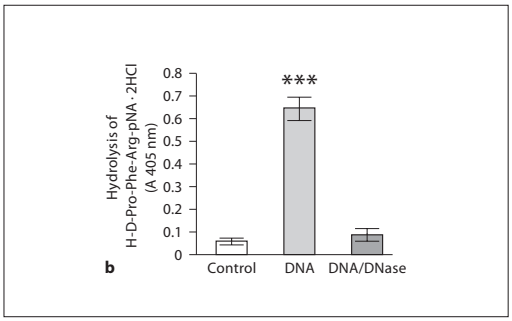
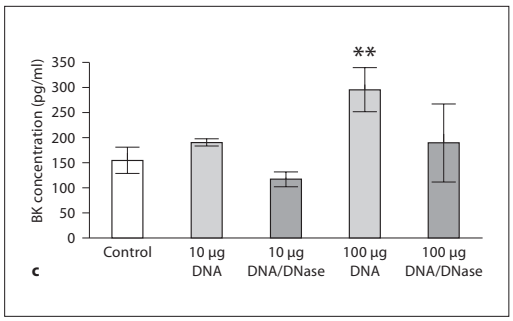


Fig. 1. Purified DNA binds and activates the contact system. **a** Transmission electron microscopy of negative-stained DNA (yellow pseudo color) HK (red pseudo color), FXII (green pseudo color) or a mixture of all 3 molecules. **b** Measurement of PK activity after incubation of plasma with purified DNA (10 μg/ml). As a control, DNA was pretreated with 200 mU DNase I for 30 min at 37°C before incubating in plasma. **c** BK release after incubation of DNA (10 or 100 μg/ml) and the DNase I-pretreated controls in human plasma for 15 min at 37°C. All Data represent the mean + SD (n = 3) of 1 representative of 3 independently performed experiments. ** $p < 0.01$; *** $p < 0.0001$. Scale bar = 10 nm.

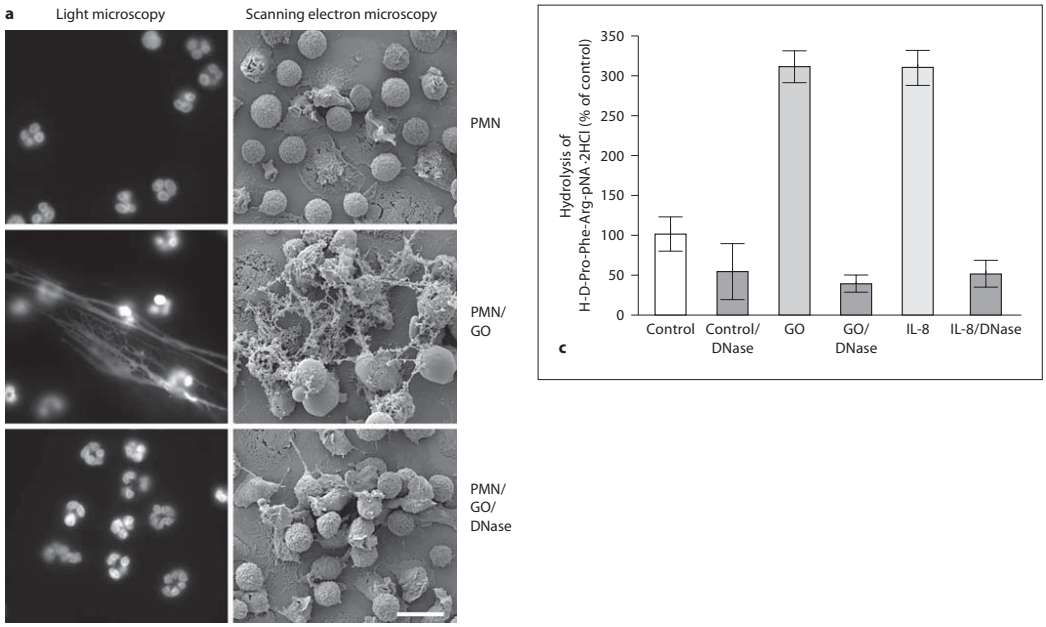




scanning electron microscopic analysis of PMNs activated with GO (fig. 2a) or IL-8 (data not shown). As expected, incubation of GO-stimulated PMNs with DNase I, led to a complete degradation of the NET structures (fig. 2a). In the next series of experiments, GO-activated PMNs were mixed with gold-labeled HK and FXII and further analyzed by transmission electron microscopy (Fig. 2b). Ultrathin sections of the stimulated PMNs revealed fine extracellular fibers, which were absent in the DNase I-treated control (fig. 2b). Furthermore, HK (small gold particles) and FXII (large gold particles) were exclusively attached to these fibers (fig. 2b) and, importantly, they were always found in close proximity to each other as seen before in experiments with soluble DNA (fig. 1a). To test whether the contact system is activated on NETs, we measured PK activity in plasma treated with nonactivated and activated PMNs. Figure 2c depicts that the addition of nonactivated PMNs to plasma did not trigger an increase in PK activity, while GO- or IL-8-stimulated PMNs induced a significant rise, which was reduced to background levels when DNase I was added to the PMNs. Based on these findings we conclude that the contact activation occurs on NET fibers.

M1 Protein/Fibrinogen Complexes Induce NET Formation

There is a growing body of evidence showing that NETs can be generated upon activation of PMNs by im-



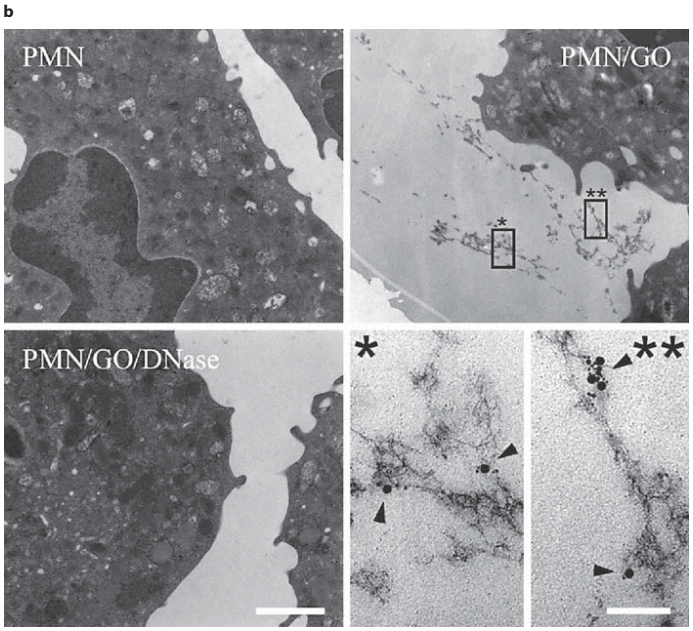


Fig. 2. NET-DNA binds contact system proteins, which is followed by activation of the contact system. **a** Light and scanning electron microscopy of nonactivated PMNs and PMNs activated with 100 mU/ml GO for 60 min. As a control, PMNs were incubated with 200 mU DNase I for 30 min after activation. Scale bar = 10 μm. **b** Transmission electron microscopy of nonactivated PMNs as well as PMNs stimulated with 100 mU/ml GO and incubated with gold-labeled HK (small gold particles) and FXII (larger gold particles). Scale bar = 1 μm. A higher magnification of 2 areas (marked with * and **) showing the binding of gold-labeled contact factors to NETs is shown on the lower right (Scale bar = 100 nm). Arrowheads point to gold-labeled proteins. PMNs incubated with 200 mU DNase I after stimulation and before adding to gold-labeled proteins are shown on the lower left. **c** PK activity after incubation of GO- and IL-8-stimulated PMNs in human plasma. Results shown are a representative of at least 3 experiments with PMNs from 3 different donors.

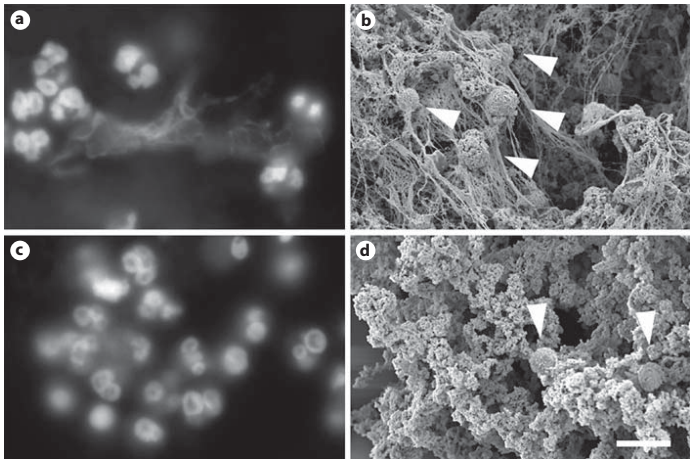


Fig. 3. NET formation after stimulating the PMNs with M1 protein/fibrinogen complexes. **a**, **b** PMNs were incubated with M1 protein/fibrinogen complexes for 60 min and investigated by light and scanning electron microscopy. **c**, **d** For light microscopy, the PMNs were stained with DAPI. As a control, the stimulated cells were treated with 200 mU DNase I for 30 min. Arrowheads point to PMNs. Scale bar = 10 μm.

portant clinical pathogens such as *Streptococcus pneumoniae, Salmonella typhimurium, Staphylococcus aureus* or *Shigella flexneri* [13, 14]. Also, lipopolysaccharide, a bacterial membrane component of Gram-negative bacteria, was proven to evoke NET formation [1]. We recently reported that soluble M1 protein of *S. pyogenes* forms complexes with fibrinogen which then trigger PMNs to mobilize their granular proteins [9]. Based on these findings, we were wondering whether this interaction also leads to the formation of NETs. PMNs were therefore incubated with M1 protein/fibrinogen complexes for 60 min and then subjected to analysis by fluorescence and scanning microscopy. Figure 3a shows that the treatment of PMNs with M1 protein/fibrinogen complexes induced a clumping of cells and the release of DNA. Analysis of the cell aggregates by scanning electron microscopy revealed that PMNs were entrapped within an amorphous precipitate built up by M1 protein/fibrinogen complexes and overlaid with NET fibers (fig. 3b). In PMN controls using M1 protein or fibrinogen alone, no aggregates or NET formation were observed (data not shown). Although DNase I treatment was able to dissolve the NET fibers, PMNs were still found in aggregates made up by M1 protein/fibrinogen complexes (fig. 3c, d).

The concept of contact activation on oligonucleotides is not entirely novel, as Kannemeier et al. [6] showed in 2007 that extracellular RNA provides a surface that allows activation of the contact system. However, we show here for the first time that extracellular DNA is also able to activate the contact system. Keeping in mind that contact activation leads to the processing of HK followed by the release of antibacterial peptides [15] and BK, a potent inflammatory mediator [for a review, see 16], it is tempting to speculate that the recruitment and activation of the contact system by NETs amplifies the innate immune response. On the other hand, streptococci may take advantage of the host defense mechanism and counteract in that they immobilize PMNs distantly from the infectious site by releasing M proteins which in concert with fibrinogen crosslink the cells and form an insoluble layer made of M1 protein/fibrinogen complexes around the PMNs. Whether or not the activation of PMNs by streptococci can neutralize the protective effect of NETs or even lead to systemic inflammatory reaction, needs to be unraveled.

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Anhang 4: Stimulation of blood mononuclear cells with bacterial virulence factors leads to the release of pro-coagulant and pro-inflammatory microparticles

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Stimulation of blood mononuclear cells with bacterial virulence factors leads to the release of pro-coagulant and pro-inflammatory microparticles

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Summary

Severe infectious diseases remain a major and life-threatening health problem. In serious cases a systemic activation of the coagulation cascade and hypovolemic shock are critical complications that are associated with high mortality rates. Here we report that blood mononuclear cells, stimulated with M1 protein of *Streptococcus pyogenes* or other bacterial virulence factors, produce not only pro-coagulant, but also pro-inflammatory microparticles (MPs). Our results also show that activation of the contact system on MPs contributes to these two effects. Phosphatidylserine (PS) plays an important role in these processes as its upregulation on MPs allows an assembly and activation of the contact system. This in turn results in stabilization of the tissue factor-induced clot and a processing of high-molecular-weight kininogen by plasma kallikrein followed by the release of bradykinin, a potent vascular mediator. Pro-coagulant monocyte-derived MPs were identified in plasma samples from septic patients and further analysis of MPs from these patients revealed that their pro-coagulant activity is dependent on the tissue factor- and contact system-driven pathway.

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Introduction

The Gram-positive bacterium *Streptococcus pyogenes* is a major human pathogen that mainly causes superficial and self-limiting skin and throat infections. However, infections can occasionally develop into serious and life-threatening conditions of which streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis are associated with high morbidity and mortality (Cunningham, 2000). Previous studies have shown that systemic contact activation plays an important role in severe *S. pyogenes* infections (Sriskandan *et al.*, 2000). The contact system – also known as the intrinsic pathway of coagulation or kallikrein/kinin system – and its activation have been extensively studied on negatively charged artificial and biological surfaces *in vitro* (Espana and Ratnoff, 1983; Tans and Griffin, 1983; Hojima *et al.*, 1984; Kannemeier *et al.*, 2007; Oehmcke *et al.*, 2009a). It consists of three plasma proteins: factor XII (FXII), plasma kallikrein (PK) and high-molecular-weight kininogen (HK). Upon activation, the contact system is involved in at least three distinct (patho)-physiologic processes: (i) activation of the intrinsic pathway of coagulation, (ii) release of bradykinin (BK) from the HK precursor by the action of PK, and (iii) generation of antimicrobial peptides (for a review see Oehmcke and Herwald, 2009).

The name 'contact system' is related to its mode of action, as binding (contact) and assembling of the contact factors to a negatively charged surface cause an activation of the system. Negatively charged phospholipids, such as phosphatidylserine (PS), are integral constituents of all eukaryotic cell membranes. Their composition varies not only among cellular organelles, but also their relative distribution within the inner and outer leaflet of a lipid bilayer can differ considerably (for a review see Leventis and Grinstein, 2010). In quiescent cells, PS is mainly found on the cytosolic side of the plasma membrane. However, upon cell activation, PS can be flipped to the outer plasma membrane, where it drives the cell to a pro-apoptotic and pro-coagulative state. Recently, the role of PS on microparticles (MPs) has attracted considerable attention.

Microparticles are sphere-shaped intact vesicles released from cell membranes of almost all activated or

apoptotic cell types. They are less than 1 μm in diameter and limited by their lipid bilayer membrane. Depending on the degree of cell activation, MPs differ in their composition and function. Notably, PS is permanently exposed on the outer membrane of the majority of MPs derived from stimulated cells. This is not observed on the cells of origin, where PS is rapidly, but transiently, externalized to the outer membrane upon a stimulatory signal (Piccin *et al*., 2007).

Phosphatidylserine plays an important role in many biological processes, for instance in apoptosis, where it acts as one major 'eat me' signal and ensures efficient recognition and uptake of apoptotic cells by phagocytes (Chaurio *et al*., 2009). A translocation of PS is particularly seen on the surface of platelets and MPs. It has been shown that PS is not only critical for the assembly of pro-coagulant complexes, but PS also amplifies the activity of tissue factor (TF), a transmembrane protein essential for the activation of the extrinsic pathway of coagulation (Butenas *et al*., 2009). Although the pro-coagulant activity of MPs has mainly been attributed to TF, little is known as to what extent the activation of the contact system is involved in these processes.

In 2006 Påhlman and colleagues reported that M protein, one of the classical virulence determinants of *S. pyogenes*, activates monocytes via the TLR2 receptor (Påhlman *et al*., 2006). These findings prompted us to study whether M proteins are able to evoke formation of pro-inflammatory and pro-coagulative MPs from peripheral blood mononuclear cells (PBMCs). Indeed, by using different experimental approaches our *in vitro* results show that the intrinsic and extrinsic pathways are activated on MPs from M1 protein-stimulated PBMCs. Interestingly, also other bacterial virulence factors stimulate the release of pro-coagulant MPs from PBMCs. Our results therefore point to an important and general role for pro-coagulant MPs in infectious diseases. In addition to these findings, we report that MP-induced contact activation contributes to clot stabilization and evokes inflammatory reactions due to the generation of BK. These findings were further confirmed *ex vivo* by studying pro-coagulant plasma MPs from septic patients, where the source of infection were caused by different bacterial pathogens including *S. pyogenes*.

Results

MPs from M1 protein-stimulated PBMCs trigger clotting via the extrinsic and intrinsic pathways of coagulation

Previous work has shown that monocytes stimulated with M proteins of *S. pyogenes* respond with the secretion of pro-inflammatory cytokines and an up-regulation of TF at their cell surface (Påhlman *et al*., 2006; 2007). To investigate, whether this treatment also triggers the release of pro-coagulant MPs, PBMCs isolated from healthy volunteers were incubated for 24 h with M1 protein of *S. pyogenes* or buffer alone (negative control). LPS from *Escherichia coli* served as a positive control (Satta *et al*., 1994). Cells were then centrifuged and the supernatants were further processed for MP isolation (see *Experimental procedures*). Trypan blue exclusion revealed that independent of the stimulus, less than 2% of the cells died within 24 h. The size distribution of the recovered MPs was in the range from 0.1 to 1 μm as determined by scanning electron microscopy (SEM, Fig. 1A). Interestingly, most particles (87%) had a size of about 0.1 μm in diameter, independent of their stimulus (Fig. 1B). Additional FACS analysis of MPs (see *Experimental procedures*) revealed that the number of MPs released from PBMCs did not increase upon stimulation with M1 protein or LPS when compared with MPs released from non-stimulated cells (data not shown).

To test the pro-coagulant activity of the MPs, equal numbers of particles were added to normal re-calcified human plasma and clotting times were determined. As shown in Fig. 1C (left panel), MPs from M1 protein- and LPS-stimulated PBMCs, triggered coagulation very efficiently (approximately 1 min), whereas the same number of MPs from non-stimulated cells failed to shorten the clotting time ($t > 17$ min). Similar results were obtained when FXII-deficient plasma was used (Fig. 1C, middle panel). These results suggest that clotting is dependent on the extrinsic pathway of coagulation. To test whether the intrinsic pathway is also involved, MPs were incubated with FVII-deficient plasma. As seen before, clotting times were reduced when MPs from M1 protein- and LPS-stimulated PBMCs were tested (Fig. 1C, right panel). However, all measuring points were approximately five-fold increased when compared with normal or FXII-deficient plasma. The effect of M1 protein- or LPS-induced MPs in FVII-deficient plasma was inhibited by co-incubation with a contact system inhibitor (HKH20) (Oehmcke *et al*., 2009b), which proofs that clotting was not triggered by a residual FVII activity in the deficient plasma. Taken together, the results show that M1 protein and LPS are equally potent inducers of pro-coagulant MPs from PBMCs. As both virulence factors signal via Toll like-receptors (Poltorak *et al*., 1998; Påhlman *et al*., 2006), we wished to test whether also other bacterial factors can trigger the formation of pro-coagulant MPs. To this end, a panel of previously described virulence determinants from Gram-positive and Gram-negative bacteria was used to generate MPs from PBMCs. Apart from M1 protein, M proteins from serotype M3 as well as the secreted protein SIC (streptococcal inhibitor of complement (Åkesson *et al*., 1996) from *S. pyogenes* and lipoteichoic acid (LTA) from *Staphylococcus aureus* were also

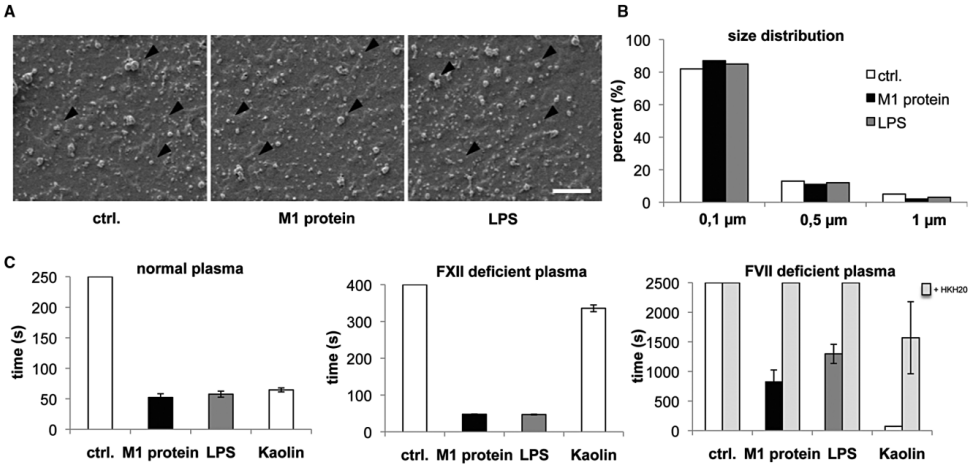

Fig. 1. Size distribution and pro-coagulant activity of PBMC MPs.
A. Scanning electron microscopy of MPs from non-stimulated (ctrl.), M1 protein- or LPS-stimulated PBMCs (scale bar 2.5 µm).
B. Size distribution of MPs from non-stimulated (ctrl.), M1 protein- or LPS-stimulated PBMCs.
C. PBMCs were stimulated with M1 protein or LPS for 24 h and MPs were purified from the supernatant. MPs isolated from non-stimulated cells were used as a control (ctrl.). MPs were adjusted to concentration of 150 MPs μl^{-1}. MPs (5 µl) or kaolin (a contact system activator) were then added to re-calcified normal plasma (left panel), FXII-deficient plasma (middle panel) or FVII-deficient plasma (right panel), and the time for clot formation was determined. The contact system inhibitor HKH20 was pre-incubated with microparticles (final concentration of 100 µM) before addition to FVII-deficient plasma, to exclude that clotting is caused by a FVII contamination.

found to release pro-coagulant MPs (Fig. S1), whereas MPs derived from protein PAB- (from *Finegoldia magna* (de Château and Björck, 1994) or protein M5-stimulated cells gave only weak response (Fig. S1). Taken together, the findings implicate that the release of pro-coagulant MPs can be triggered by other bacterial virulence factors as well, and is not restricted to M proteins of *S. pyogenes*.

Clot structure and endurance against fibrinolysis

Our findings show that pro-coagulant MPs activate both pathways of the coagulation cascade, and suggest that the two arms of the coagulation system have different functions during clot formation. We therefore set up a series of experiments to test whether the extrinsic pathway is important in initiating clotting, while intrinsic pathway contributes to the clot morphology and stability. MPs from M1 protein-stimulated PBMCs were added to re-calcified normal, FVII- or FXII-deficient plasma. The generated clots were fixed immediately (termed as 1 min clot) or 10 min (10 min clot) after clot formation, which should resemble the situation before and after contact activation. Samples were then subjected to morphological analysis by light and scanning electron microscopy. Clots that were processed immediately (1 min clots) appeared as a wide-meshed fibrin network and no differences were observed regardless of whether normal or FXII-deficient plasma was used (data not shown). Note that no clotting was observed in re-calcified FVII-deficient plasma after 1 min. We next analysed 10 min clots that were formed in normal plasma. Microscopic analysis revealed that clots were built up by a dense network, consisting of long and thick fibrin strands that are tightly assembled (Fig. 2A, upper panel). This was not seen with FXII- and FVII-deficient plasma, where the clot architecture was looser with thinner fibrin strands (Fig. 2A, middle panel) or formed by thick and short fibrin strands arranged in a diffuse manner (Fig. 2A, lower panel) respectively.

The differences in the clot morphology were also reflected in their resistance to breakdown by tissue-type plasminogen activator (tPA). To this end, clots were generated as described above and subjected to fibrinolysis by the addition of tPA. Figure 2B shows that 1 min clots produced from normal plasma were dissolved within 8.6 min, while lysis time for 10 min clots was almost doubled (17 min). To test whether inhibition of the contact system leads to the formation of less stable clots, HKH20 was added to normal plasma. We found that this treatment shortened tPA-induced lysis of the 1 min clots to 5 min and also the lysis time of HKH20 treated 10 min clots was decreased (from 17 to 8.5 min). The latter lysis time was in the same range as the lysis time for the 1 min clots of

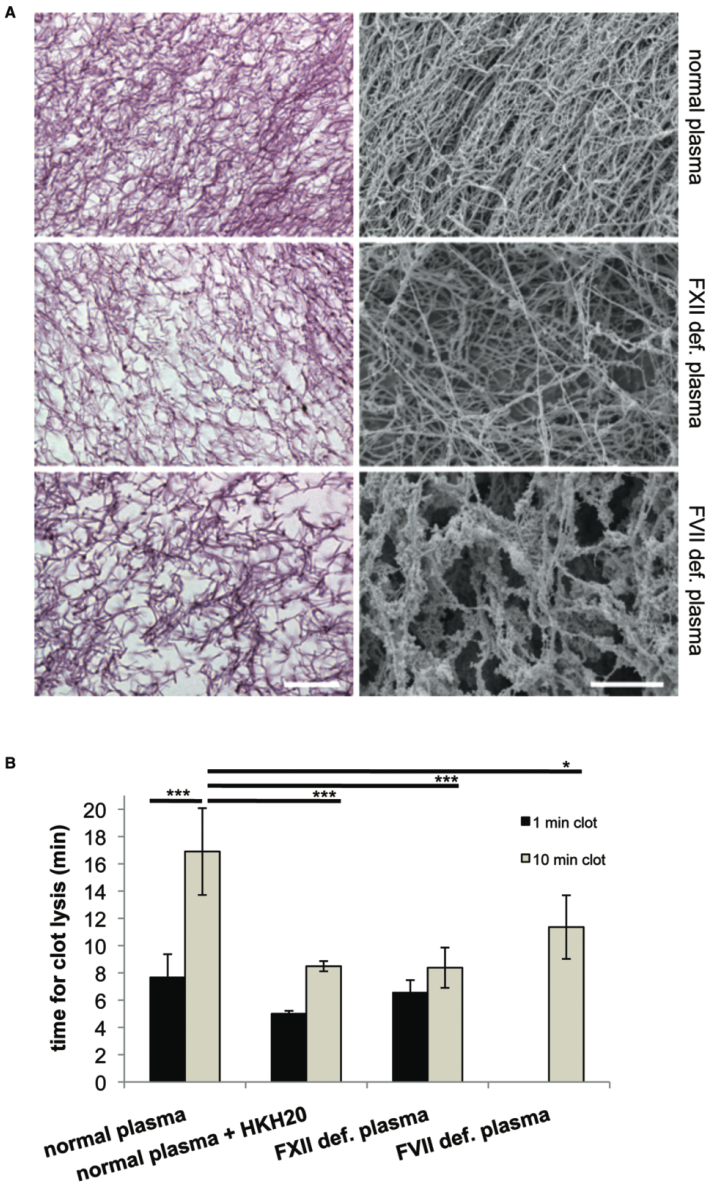

Fig. 2. Clot structure and clot lysis.
A. Clots were induced by addition of MPs from M1 protein-stimulated PBMCs to re-calcified plasma, incubated for 10 min, fixed and processed for light microscopy (left panel, HE stain, scale bar 10 μm) or scanning electron microscopy (right panel, scale bar 10 μm).
B. Clots were generated as described above and tPA were added immediately (1 min clot) or 10 min after (10 min clot) re-calcification. The times for clot lysis were measured in a coagulometer. $^{*}P < 0.05$, $^{***}P < 0.0001$.

 Cellular Microbiology, **14**, 107–119

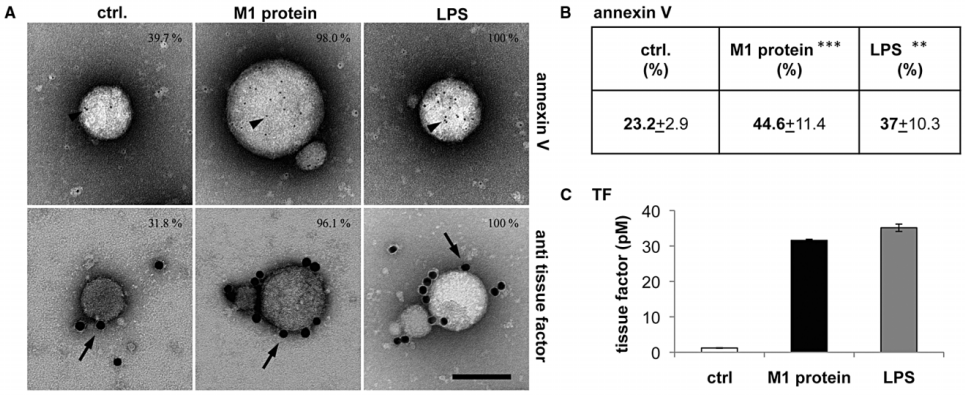

Fig. 3. TF and PS exposure on PBMC-derived MPs.
A. MPs derived from non-stimulated (ctrl.), M1 protein- or LPS-stimulated PBMCs were incubated with a gold-labelled antibody against TF (15 nm gold, lower panel), or gold-labelled annexin V (5 nm, upper panel), processed by negative staining and analysed in a transmission electron microscope. Arrows point to gold-labelled antibody against TF and arrowheads to gold-labelled annexin V, scale bar 100 nm.
B. Measurement of annexin V binding by FACS analysis. MPs were incubated with annexin V and 5000 events were counted and analysed. Positive events are presented as percentage of 5000 events, $^{**}P < 0.005$, $^{***}P < 0.0001$.
C. Quantification of TF pro-coagulant activity of MPs (150 MPs μl^{-1}) by ELISA.

non-HKH20 treated normal plasma, suggesting that inhibition of the contact system by HKH20 impairs clot stability. Similar findings were also recorded when FXII-deficient plasma was used. Using this approach we observed that the 10 min clots dissolved almost at the same time as the 1 min clots made from normal plasma. Finally, we measured the lysis of clots generated from FVII-deficient plasma. Here, we found that the time for clot lysis was prolonged when compared with normal plasma treated with HKH20 or FXII-deficient plasma (10 min clots). Taken together, the tPA experiments show that an impaired contact system leads to accelerated clot lysis.

TF and PS composition is altered in MPs derived from stimulated PBMCs

In order to activate both coagulation pathways, MPs have altered to a pro-coagulant surface. Exposure of PS on the outer leaflet of the membrane from MPs has been reported to be a critical component in this process (Key, 2010) and, thus, gold-labelled annexin V was employed to determine PS translocation on MPs. Examination and quantification by transmission electron microscopy (TEM) revealed gold-labelled annexin V binds more abundantly to MPs from stimulated PBMCs (Fig. 3A, upper panel) and similar findings were recorded when MPs were analysed by FACS (Fig. 3B). We next studied the distribution of TF on PBMC-derived MPs. Figure 3A depicts that gold-labelled antibodies against TF bound more frequently to MPs from M1 protein or LPS treated cells than to MPs from non-stimulated cells (Fig. 3A, lower panel). The ELISA measurements also showed that TF significantly increased on MPs derived from stimulated PBMCs (Fig. 3C). Collectively, the data show that the pro-coagulant state of MPs from stimulated PBMCs is primed by translocation of PS to the outer membrane in combination with concurrent increase of TF content.

MPs bind and activate the contact system

We next studied the effect of pro-coagulant MPs on the contact system. To this end, MPs from stimulated PBMCs were mixed with gold-labelled HK and FXII, while MPs from non-stimulated PBMCs served as control. MPs were also stained with gold-labelled CD14 antibodies, to determine monocytic origin of the particles. Negative staining and analysis by TEM visualized HK (small gold particles) and FXII (middle sized gold particles) that were most frequently attached to MPs from M1 protein- and LPS-stimulated CD14-positive monocytes (big sized gold particles) and to a lesser extent to MPs from non-stimulated monocytes (Fig. 4A). To test whether the binding of contact factors to MPs is followed by an activation of the contact system, we measured PK activity and BK release in plasma treated with equal amounts of MPs from non-stimulated and stimulated PBMCs. Figure 4B depicts that the addition of MPs from non-stimulated PBMCs to plasma did not trigger an increase of PK activity, while MPs from M1 protein- or LPS-stimulated cells induced a

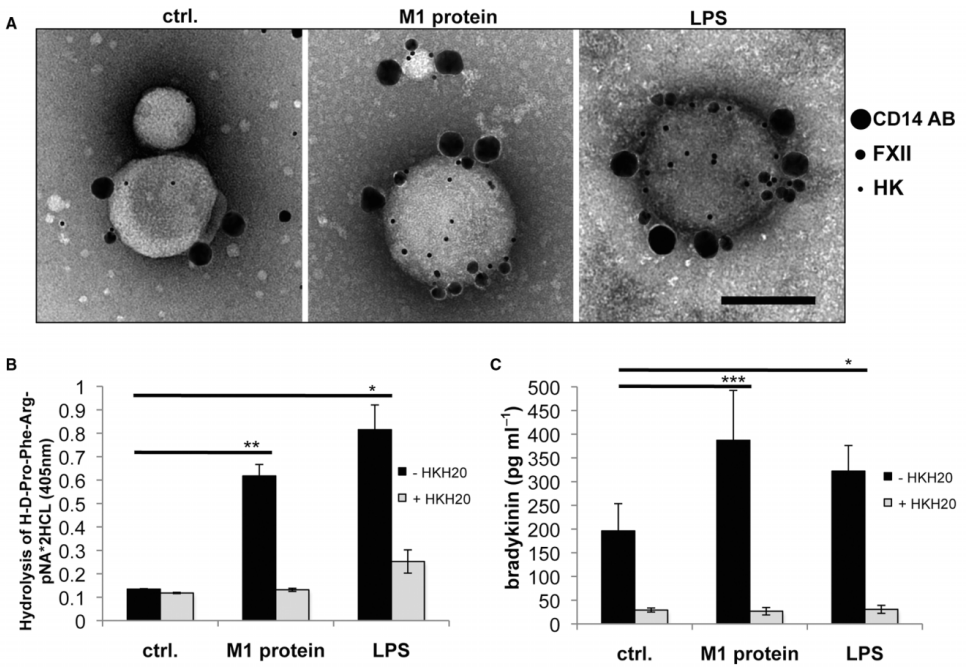

Fig. 4. Binding and activation of the contact system on MPs.
A. MPs derived from non-stimulated (control), M1 protein- or LPS-stimulated PBMCs were incubated with gold-labelled FXII (15 nm gold), gold-labelled HK (5 nm gold), and gold-labelled anti-CD14 antibody (30 nm gold). CD14-positive MPs were quantified and their FXII and HK content was determined.
B. Plasma kallikrein activity was recorded in plasma 30 min after incubation with MPs derived from non-stimulated, M1 protein-stimulated (black column), or LPS-stimulated PBMCs (150 MPs μl^{-1}) in a substrate assay. Contact activation was blocked by the addition of the contact system inhibitor HKH20 (light grey columns), $^{*}P < 0.05$, $^{**}P < 0.005$.
C. Bradykinin release was measured in plasma 30 min after incubation with MPs derived from non-stimulated, M1 protein-stimulated (black column), or LPS-stimulated PBMCs (100 MPs μl^{-1}) and HKH20 was added to prevent bradykinin formation (light grey columns). The results are representative of at least three experiments performed in triplicates, $^{*}P < 0.05$, $^{***}P < 0.0001$.

significant rise. Addition of HKH20 to the MPs before incubation with plasma reduced PK activity to background levels. Likewise, BK release was also significantly increased, when plasma was incubated with MPs from stimulated, but not from non-stimulated cells (Fig. 4C). As seen before, this effect was blocked, when MPs were co-incubated with HKH20 (Fig. 4C). These results show that MPs from stimulated PBMCs are potent activators of the contact system.

PS triggers the release of bradykinin

Earlier studies demonstrated that acidic phospholipids such as PS promote binding and activation of the contact system in plasma (Schousboe, 1988). To further prove whether this activation triggers BK release, we generated vesicles consisting of pure PS, while vesicles made of the non-charged phosphatidylcholin (PC) served as negative control. The vesicular nature of both phospholipids in solution was confirmed by negative staining and TEM. Subsequent electron microscopic analysis revealed that gold-labelled FXII and HK avidly bind to PS, but not to PC vesicles (Fig. 5A, upper panel). Similar findings were also recorded when gold-labelled PS – binding annexin V was used as a positive control (Fig. 5A, lower panel). Based on these findings, we tested whether PS is able to trigger an activation of the contact system. PS and PC vesicles were incubated with normal human plasma and the induction of PK activity was measured. Figure 5B shows that the addition of PS vesicles to plasma leads to a time-dependent rise in plasma kallikrein activity, which was not seen with PC vesicles. Moreover, a significant BK release was measured, when PS, but not PC, vesicles were incubated with plasma (Fig. 5C). These data suggest that an

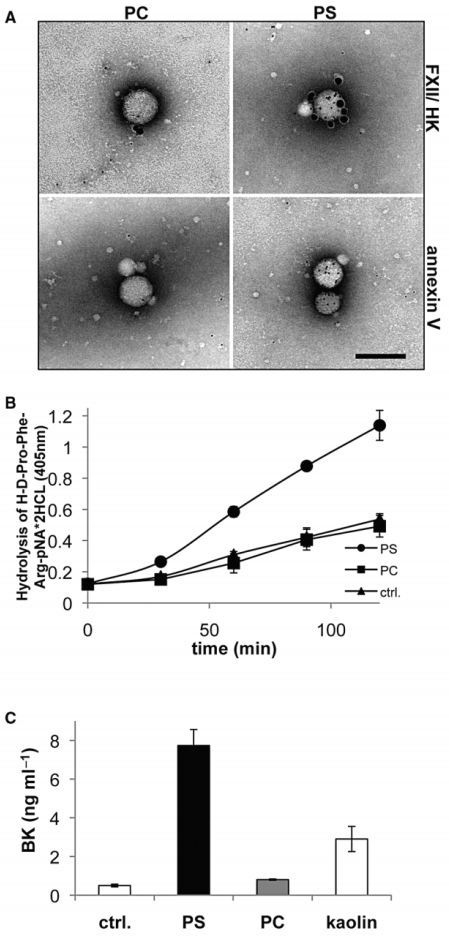

Fig. 5. Binding and activation of the contact system on PS vesicles.
A. PS and PC vesicles were incubated with gold-labelled HK and FXII (5 nm gold and 15 nm gold respectively; upper panel) or gold-labelled annexin V (5 nm; lower panel). The vesicles were then processed by negative staining and analysed in a transmission electron microscope. Scale bar 100 nm.
B. PS and PC vesicles at a final concentration of 100 nM or buffer were incubated with normal human plasma for 2 min before a substrate, specific for plasma kallikrein activity, was added. After indicated time points the absorbance in the reaction mixture was measured at 405 nm.
C. PS, PC, kaolin (positive control) or buffer (ctrl.) were incubated with normal human plasma for 30 min followed by measurements of the BK content by ELISA.

increase in the PS content on MPs promotes the assembly and activation of the contact system and is followed by the release of BK.

The concentration of PS-positive MPs is increased in plasma samples from septic patients

To address whether contact activation can be monitored on plasma MPs from septic patients, we analysed plasma samples from 14 septic patients (including 4 samples from patients suffering from a streptococcal sepsis). The activated partial thromboplastin time (aPTT), as a marker for the intrinsic pathway of coagulation, and the prothrombin time (PT), as a marker for the extrinsic pathway of coagulation, were significantly prolonged in all septic patients, when compared with healthy volunteers (Fig. 6A). On average, the total numbers of plasma MPs were higher in septic patients; however, the difference was not statistically significant (Fig. 6A, right panel).

When measuring the content of PS-positive plasma MPs from sepsis patients (Fig. 6B), we found that PS levels were in the range from 6 to 68 nM (a PS concentration above 10 nM is considered as pathologic), whereas the PS levels in all control samples were below 3 nM. Additional statistical analysis of the MP and PS content in these samples revealed a significant positive correlation (Fig. S2A, $r = 0.77$). Thus, the data show that the content of PS-positive MPs reaches pathological levels in the plasma of septic patients. This in turn equips the MPs with a pro-coagulant surface and may further promote activation of the contact system and generation of bradykinin.

Plasma MPs from septic patients activate both pathways of coagulation

To dissect the influence of the extrinsic and intrinsic pathway of MPs from septic patients, a series of clotting assays was performed. MPs purified from 200 µl plasma were used undiluted in clotting assays (see *Experimental procedures*). When the MPs were added to normal human plasma, we observed that MPs from seven septic patients (three from *S. pyogenes* infected patients) caused significantly shorter clotting times as compared with those from healthy volunteers (Fig. 6C, septic patient group 1). Importantly, MPs from these patients also decreased the clotting times in FXII- or FVII-deficient reconstituted plasma (Fig. 6C), suggesting that both pathways contribute to the pro-coagulative activity. However, MPs from sepsis patients that had no effect in human plasma (group 2) were in general also not able to affect the clotting times in FXII- or FVII-deficient plasma (Fig. 6C). Statistical analysis showed a significant negative correlation between the increase in the PS content and the

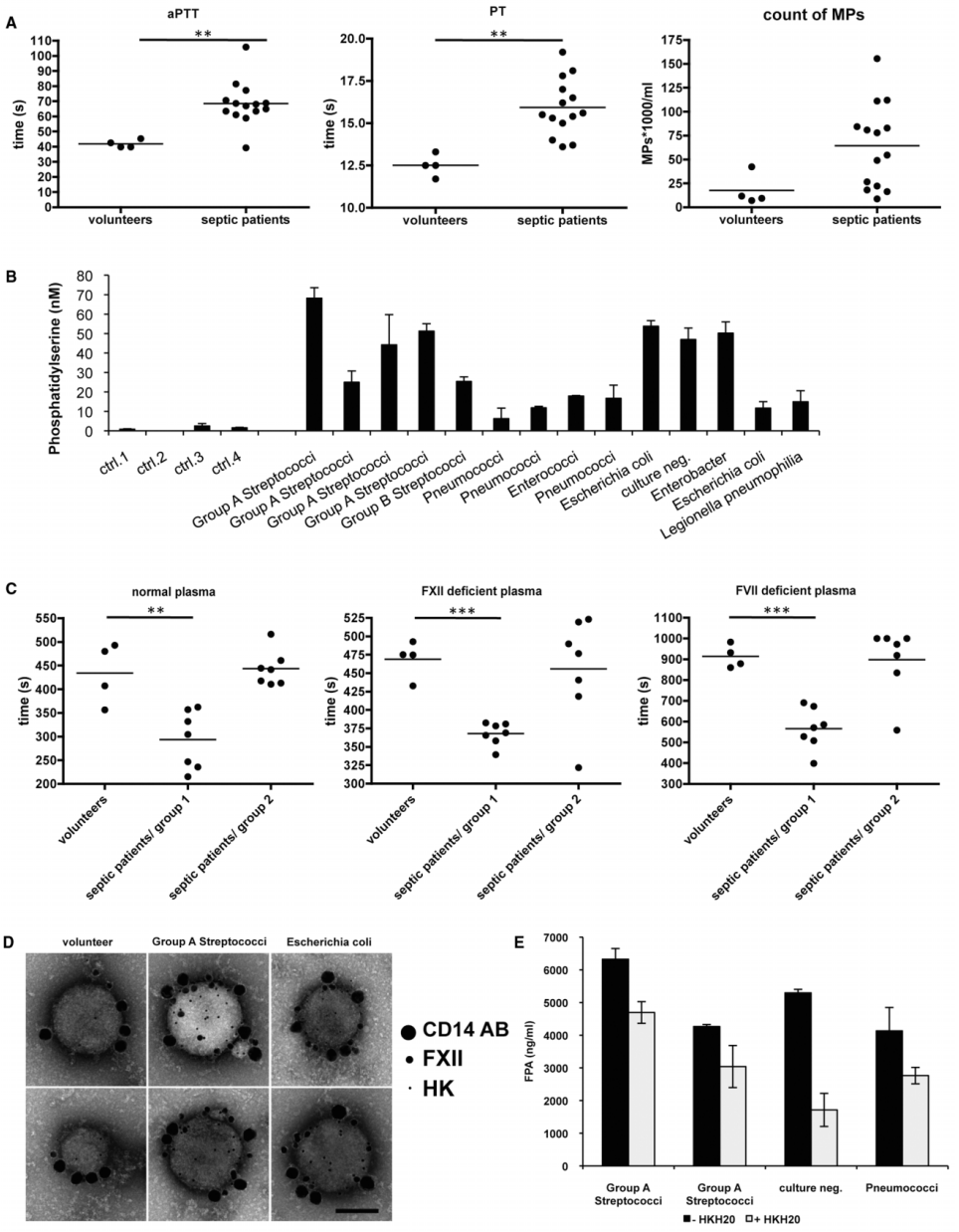
A
aPTT
**
time (s)
volunteers
septic patients
PT
**
time (s)
volunteers
septic patients
count of MPs
MPs*1000/ml
volunteers
septic patients
B
Phosphatidylserine (nM)
ctrl.1
ctrl.2
ctrl.3
ctrl.4
Group A Streptococci
Group A Streptococci
Group A Streptococci
Group A Streptococci
Group B Streptococci
Pneumococci
Pneumococci
Enterococci
Pneumococci
Escherichia coli
culture neg.
Enterobacter
Escherichia coli
Legionella pneumophilia
C
normal plasma
**
time (s)
volunteers
septic patients/ group 1
septic patients/ group 2
FXII deficient plasma
***
time (s)
volunteers
septic patients/ group 1
septic patients/ group 2
FVII deficient plasma
***
time (s)
volunteers
septic patients/ group 1
septic patients/ group 2
D
volunteer
Group A Streptococci
Escherichia coli
CD14 AB
FXII
HK
E
FPA (ng/ml)
Group A Streptococci
Group A Streptococci
culture neg.
Pneumococci
- HKH20
+ HKH20

Fig. 6. Analysis of plasma and plasma-derived MPs from septic patients.
A. Measurement of clotting times and MP content in plasma samples from healthy volunteers and septic patients, $^{**}P < 0.005$.
B. Measurement of pro-coagulant activity of annexin V positive MPs in plasma. Results were expressed as PS equivalent (nM).
C. MPs isolated from plasma of healthy persons and septic patients were added to fresh re-calcified normal plasma, FXII- or FVII-deficient plasma and the clotting times were determined, $^{**}P < 0.005$, $^{***}P < 0.0001$.
D. Plasma MPs derived from two healthy volunteers and four septic patients were incubated with gold-labelled FXII (15 nm gold), gold-labelled HK (5 nm gold), and gold-labelled anti-CD14 antibody (30 nm gold). Representative pictures from one volunteer and two septic patients are shown.
E. Measurement of fibrinopeptide A (FPA) in normal human plasma, 20 min after addition of MPs derived from plasma of septic patients. Samples were also treated with HKH20 (100 μM) to prevent an activation of the contact system.

clotting time, when MPs from healthy volunteers and septic patients were analysed (Fig. S2B). This correlation was even more significant when MPs were incubated with FVII deficient plasma (Fig. S2C), suggesting that activation of the contact system is dependent on the PS content. By contrast, the reconstitution of MPs with FXII deficient plasma was not significantly dependent on the PS content (Fig. S2D). These findings point to an important role of PS in activating the contact system on MPs, but not for the extrinsic pathway of coagulation.

To test whether monocyte-derived MPs can be detected in plasma samples from septic patients, MPs from four patients with high pro-coagulant MPs (three patients with a *S. pyogenes* sepsis) were incubated with CD14 antibodies for analysis by FACS and TEM. In all four samples 5–6% CD14-positive MPs were found, while only less than 1% of MPs from healthy volunteers were of monocyte origin (data not shown). Additional triple immunogold staining and TEM revealed that CD14-positive MPs from all septic patients were able to bind gold-labelled FXII and HK on their surface (Fig. 6D).

Fibrinopeptide A (FPA) is a 16-amino-acid peptide released from the amino terminus of fibrinogen Aα chains by thrombin cleavage. Elevated levels of FPA indicate an increase in fibrin network formation and, thus, measurements of FPA present a sensitive method to determine fibrinogen conversion to fibrin and subsequent clot formation. When MPs isolated from two healthy volunteers were added to normal re-calcified plasma, the FPA levels in the samples was below the detection level (not shown). This was in contrast to MPs from the four septic patients (see above) where a massive release of FPA was seen when subjected to the same experimental protocol (Fig. 6E). When the experiments were performed in the presence of HKH20 a drop in the FPA content in all four septic samples was measured. These results imply that fibrinogen conversion to fibrin and subsequent fibrin network formation are amplified upon contact activation, which in turn may affect clot stability (Fig. 6E). Taken together, the data show that pro-coagulant monocyte-derived MPs are found in the plasma from septic patients. In addition, our results suggest that pro-coagulant MPs have the ability to activate both arms of the coagulation system, which in a concerted manner can create a stable clot.

Discussion

Microvascular thrombosis is a hallmark in infectious diseases (van Gorp *et al*., 1999), and it can, under systemic conditions, contribute to serious and life-threatening complications. When activated locally, however, microvascular thrombosis can be also part of the immune response for instance by preventing bacterial spreading (Dixon, 2004). Both scenarios (systemic versus local) depend on a coordinated thrombus formation and *in vivo* studies have demonstrated an important role for MPs in these processes (Furie and Furie, 2005). When looking at the molecular mechanisms involved, it was found that pro-coagulant MPs can be incorporated into a growing thrombus in a P-selectin-dependent manner. This will then trigger thrombin generation and fibrin clot propagation via TF pathway (Falati *et al*., 2003). These findings suggest that MPs exposing TF play a central role in the initiation of coagulation under pathological conditions (Nomura *et al*., 2008).

Streptococcus pyogenes is one of the most important pathogens encountered worldwide (Carapetis *et al*., 2005) and it was estimated by the WHO that severe streptococcal infections cause the death of at least 517 000 patients, annually (Carapetis *et al*., 2005). The pathogenesis of invasive infections with this microorganism is often combined with a systemic activation of the coagulation system and an overwhelming inflammatory response. Either of the two host responses is considered to contribute to deleterious conditions during the course of disease. Among the different streptococcal virulence factors studied, M proteins have attracted considerable attention, since they can evoke pathologic pro-coagulative and pro-inflammatory reactions by targeting different cell types such as monocytes, neutrophils, and T-cells (Oehmcke *et al*., 2010). The aim of this study was to examine whether M protein can in addition evoke systemic responses by the generation of MPs, a highly mobile carrier of pro-coagulative and pro-inflammatory activity. A special focus was set on MPs derived from monocytes, since monocytes are thought to be the major source of TF positive MPs (Owens and Mackman, 2011).

Our results show that purified blood mononuclear cells, stimulated with M1 protein, release pro-coagulant

TF positive MPs, and also virulence factors from other bacterial pathogens were found to have similar activities. These findings point to a general patho-physiological role of pro-coagulant MPs in infectious diseases. Our data also show that the pro-coagulant activity of MPs is triggered by the extrinsic pathway in concert with activation of the intrinsic pathway. Notably, other bacterial virulence factors apart from M1 protein are also able to release pro-coagulant MPs from PBMCs, suggesting that the mechanisms described here are of general nature and not restricted to infections caused by *S. pyogenes*.

Our results raise the question about the pathophysiological relevance of MPs in their ability to activate the extrinsic and the intrinsic pathways of coagulation. It is noteworthy that less than a decade ago it was generally accepted that the contact system only plays a secondary role in hemostasis, because patients with deficiencies in FXII, PK or HK normally do not suffer from bleeding disorders. The point of view has, however, changed when Renné and colleagues reported in 2005 that the contact system contributes to the formation and stabilization of three-dimensional thrombi (Renné *et al*., 2005). Taking these findings in consideration it seems plausible that pro-coagulant MPs induce thrombus formation via the TF pathway, followed by an activation of the intrinsic pathway that helps to stabilize the formed clot. As a low concentration of contact factors correlates with a fatal outcome of the disease (Pixley and Colman, 1997), it is tempting to speculate that patients with an increased number of procoagulative MPs and low contact factors levels will form non-stable clots that can end-up as microthrombi in the circulation. This condition, also referred as disseminated intravascular coagulation, is often seen in patients with systemic coagulation dysfunction and is associated with high mortality rates.

Having a closer look at the molecular mechanisms that lead to an activation of the contact system on MPs, we established an important role for PS as a docking and activation site for contact factors. The importance of PS as activator of the contact system has been controversially discussed in the literature. For instance Schousboe (1988) found amidolytic activity of contact factors when PS was added to plasma, while Rojkjaer and Schousboe (1997) reported that FXII binds to, but does not become activated on PS vesicles. Our study is in line with the first report and we show a critical role of PS as activator of the contact system, leading to an increased PK activity and the release of BK.

BK release by contact system activation is another example showing how tightly hemostasis and inflammation are linked together. Here we report for the first time that BK is released from MPs. Kinins are potent inducers of vascular leakage, hypovolemic hypotension and oedema formation, which are critical features in severe bacterial infections.

Taken together, the data presented here show an important role for the contact system on pro-coagulant MPs. Our findings also support the notion that this mechanism contributes to clot stabilization and the induction of inflammatory reactions. As hemostasis and inflammation are tightly linked processes, a better understanding of the bacterial virulence mechanisms that target both cascades may lead to novel therapeutic concepts in infectious diseases.

Experimental procedures

M1 protein, M3 protein, M5 protein, peptostreptococcal albumin-binding protein (PAB) and protein SIC were purified described before (Åkesson *et al*., 1996; Collin and Olsén, 2000; Påhlman *et al*., 2006). LPS and LTA were obtained from Sigma-Aldrich (USA). Deficient plasmas were from George King Bio-Medical (Overland Park, KS, USA). Plasmas from septic patients were from the University Hospital, Lund and Malmö, Sweden. The project protocol was approved by the ethics committee of Lund University Hospital, and informed consent was obtained from all patients or their close relatives. Blood was transferred to plastic tubes and centrifuged (2000 *g* for 10 min). To obtain platelet free plasma (PFP) samples were centrifuged (10 000 *g* for 5 min) and supernatants were frozen at –80°C. HKH20 (HKHGHGHGKH-KNKGKKNGKH) was produced as described (Oehmcke *et al*., 2009b).

Preparation of MPs from PBMCs or plasma

Peripheral blood mononuclear cells were isolated from human citrated blood from healthy volunteers as described (Påhlman *et al*., 2007). The PBM cell layer was collected; cells were washed twice in PBS and resuspended in RPMI medium (Invitrogen). PBMCs (2×10^6 cells ml^{-1}) were treated with different M proteins (1 μg ml^{-1}), LPS (100 ng ml^{-1}), LTA (1μg ml^{-1}) or medium alone (final volume of 1000 μl). After an overnight incubation on rotation at 37°C, cells were centrifuged (400 *g* for 20 min). Supernatants were kept frozen (–80°C) until use. Samples (900 μl supernatant or 200 μl plasma) were thawed at 37°C and centrifuged (20 817 *g* for 30 min) at room temperature to pellet the MPs. Supernatants were removed and 900 μl of a buffer containing 15 mM Hepes (ICN Biomedicals, Aurora, OH, USA), and 135 mM NaCl (Hepes buffer) were added. MPs were pelleted by centrifugation, 800 μl of the supernatant were removed, and the MPs were resuspended in the remaining 100 μl buffer.

Flow cytometric analysis of plasma samples and MPs

To identify the cellular origin of MPs, 10 μl purified MPs were mixed with 30 μl Hepes buffer containing 2.5 mmol $CaCl_2$ and incubated with 5 μl PE-labelled and 5 μl FITC-labelled monoclonal antibody (mAb) for 20 min. The following antibodies and proteins were used: 100 μg ml^{-1} IgG1-FITC isotype control mouse (Antibodies-online GmbH, Aachen, Germany), IgG1-PE isotype control mouse (Antibodies-online GmbH, 100 μg ml^{-1}), 60 μg ml^{-1} anti-CD14-PE

(Dako, Glostrup, Denmark), 100 μg ml^{-1} anti-TF-FITC (American Diagnostica, Stamford, CT, USA), and 5 μl annexin V-PE (Invitrogen, Carlsbad, CA, USA). The incubation was terminated by adding 500 μl Hepes buffer containing 2.5 mmol l^{-1} $CaCl_2$. Samples were analysed in a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA, USA). Both forward scatter and sideward scatter were set at a logarithmic gain. MPs were identified on forward scatter, sideward scatter and by binding of annexin V and cell-specific mAbs. Non-specific binding and auto-fluorescence were corrected by subtracting the fluorescence signal of the control IgG antibodies. For all experiments the number of MPs were determined by using a fluorescent bead count based assay (Orozco and Lewis, 2010). Ten microlitres of PFP or purified MPs was mixed with 10 μl Rainbow Calibration Particles (BD Biosciences, 6–6.4 μm) and 550 μl Hepes buffer. Due to their size, Rainbow Calibration Particles can be easy differentiated from MPs when analysed by light scatter. The Rainbow Calibration Particles (200 μl^{-1}) were counted (1000 events) on a separate gate and the number of MPs was calculated.

Clotting experiments

Clotting times were measured using an Amelung coagulometer as described before (Oehmcke *et al.*, 2009b). To test the pro-coagulant effect of MPs, 50 μl of human citrated plasma was reconstituted with 50 μl 30 mM $CaCl_2$ and 5 μl washed MP suspensions was added (concentration 50–150 MPs μl^{-1}). The time for clot formation was determined. Alternatively, FXII- or FVII-deficient plasma was used.

Clot lysis

Clots were generated with MPs from M1 protein-stimulated PBMCs as described above. After 1 and 10 min, 25 μl Actilyse (1 mg ml^{-1}, Boehringer Ingelheim GmbH, Germany) were added to the clot and resuspended. The time to clot lyses was measured in the Amelung coagulometer.

Phospholipid preparation

PC and PS (Sigma-Aldrich, USA) liposomes were prepared by adding the phospholipids (100 μM) to Eppendorf tubes. Samples were centrifuged in a SpeedVac to evaporate chloroform. Hepes buffer was added, the mixture was vortexed for 5 min, and subjected to 10 cycles of ultrasonication (Hielscher Ultrasound Technology, Teltow, Germany).

Chromogenic substrate assay

Microparticles were resuspended (150 MPs μl^{-1}) in Hepes buffer. Alternatively phospholipids were prepared as described above and diluted to a concentration of 100 μM. Ten microlitres of citrated plasma was incubated with 10 μl MPs, or 1 μl phospholipids (PS or PC), or 10 μl Hepes buffer (negative control) for 2 min and added to 100 μl Hepes containing 500 μM $ZnCl_2$ and 1 mM chromogenic substrate S-2302 (Chromogenix, Milano, Italy). After 15, 30 and 60 min of incubation at 37°C, the absorbance was measured at 405 nm in an ELISA reader.

Bradykinin assay

BK contents were determined by ELISA as described (Mattsson *et al.*, 2001). MPs and phospholipids were prepared and diluted as described (chromogenic substrate assay).

TF activity ELISA

The Actichrome TF activity assay kit (American Diagnostica) was used to quantify the TF pro-coagulant activity in the MP samples.

Measurement of MP pro-coagulant activity

The ACTICHROME Microparticle Activity kit (American Diagnostica) was used according to the instructions of the manufactory, to measure the pro-coagulant activity of MPs. Results were expressed as PS equivalent (nM) by employing a standard curve with liposomes of known PS concentrations from the kit. The system does not allow the capture of lipoproteins, and the eventual presence of TF on captured MP does not alter values corresponding to PS content, as it is based on a prothrombinase assay (Jy *et al.*, 2004).

Measurement of fibrinopeptide A (FPA) release

To investigate the release of FPA in human plasma treated with MPs, the IMUCLONE FPA ELISA (American Diagnostica) was used. Ten microlitres of plasma was re-calcified with 10 μl $CaCl_2$ (30 mM) and 2–5 μl of plasma MPs were added. Controls were made with buffer instead of MPs. In some samples HKH20 (100 μM; final concentration) were added. After 20 min of incubation samples were treated twice with 10 μl Bentonite to remove any cross-reactive fibrin(ogen). Samples were kept frozen (–20°C) until ELISA processing. Before usage samples were diluted 100 times in sample dilution buffer.

Histopathological evaluation of clots

Clots were generated as described above and fixed at room temperature for 24 h in buffered 4% formalin (pH 7.4; Kebo). Processing for histopathology occurred as described (Oehmcke *et al.*, 2009b).

Scanning electron microscopy

Clot samples were fixed as previously described (Oehmcke *et al.*, 2009b). Samples were processed and sputtered with palladium/gold as described (Oehmcke *et al.*, 2009a) and examined in a Jeol JSM-350 scanning electron microscope.

Negative staining and transmission electron microscopy

Human proteins (HK, FXII, anti-CD14 AB, and anti-TF AB) were labelled with colloidal gold (30, 15 and 5 nm in diameter, BBI International) as described earlier (Bengtson *et al.*, 2006). MPs or phospholipids were mixed with gold-labelled 20 nM proteins for 20 min at room temperature and processed for negative staining (Bober *et al.*, 2010).

Statistical analysis

Statistical analysis was performed using GraphPadPrism 4.00. The *P*-value was determined by using the unpaired *t*-test. All samples were analysed in triplicate and experiments were performed at least three times. The bars in the figures indicate standard deviation.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. PBMCs were stimulated with M1 protein, M3 protein, M5 protein, protein SIC, protein PAB (1 μg ml^{-1} each protein), LPS (100 ng ml^{-1}), or LTA (1 μg ml^{-1}) for 24 h and MPs were purified from the supernatant. MPs isolated from non-stimulated cells were used as control (ctrl.). Samples were adjusted to a concentration of 50 MPs μl^{-1}. Five microlitres of each preparation was then added to re-calcified normal plasma and the time for clot formation was determined.
Fig. S2. A. Positive significant correlation between number of plasma MPs and PS content in plasma samples from healthy volunteers (○) and septic patients (■).
B. Negative significant correlation between clotting times of the MPs (in normal plasma) and PS concentration in plasma samples from healthy volunteers (○) and septic patients (■).
C. Negative significant correlation between clotting times of the MPs (in FVII-deficient plasma) and PS concentration in plasma samples from healthy volunteers (○) and septic patients (■).
D. No significant correlation between clotting times of the MPs (in FXII-deficient plasma) and PS concentration. Pearson's correlation was used for statistic analysis.

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Anhang 5: A Novel Role for Pro-Coagulant Microvesicles in the Early Host Defense against *Streptococcus pyogenes*

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A Novel Role for Pro-Coagulant Microvesicles in the Early Host Defense against *Streptococcus pyogenes*

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Abstract

Previous studies have shown that stimulation of whole blood or peripheral blood mononuclear cells with bacterial virulence factors results in the sequestration of pro-coagulant microvesicles (MVs). These particles explore their clotting activity via the extrinsic and intrinsic pathway of coagulation; however, their pathophysiological role in infectious diseases remains enigmatic. Here we describe that the interaction of pro-coagulant MVs with bacteria of the species *Streptococcus pyogenes* is part of the early immune response to the invading pathogen. As shown by negative staining electron microscopy and clotting assays, pro-coagulant MVs bind in the presence of plasma to the bacterial surface. Fibrinogen was identified as a linker that, through binding to the M1 protein of *S. pyogenes*, allows the opsonization of the bacteria by MVs. Surface plasmon resonance analysis revealed a strong interaction between pro-coagulant MVs and fibrinogen with a K_D value in the nanomolar range. When performing a mass-spectrometry-based strategy to determine the protein quantity, a significant up-regulation of the fibrinogen-binding integrins CD18 and CD11b on pro-coagulant MVs was recorded. Finally we show that plasma clots induced by pro-coagulant MVs are able to prevent bacterial dissemination and possess antimicrobial activity. These findings were confirmed by *in vivo* experiments, as local treatment with pro-coagulant MVs dampens bacterial spreading to other organs and improved survival in an invasive streptococcal mouse model of infection. Taken together, our data implicate that pro-coagulant MVs play an important role in the early response of the innate immune system in infectious diseases.

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Introduction

Today it is generally accepted that coagulation is tightly interwoven with the innate immune system [1]. Both systems can act in a combined effort to sense and eradicate an infection in a highly sophisticated manner. Indeed, evolutionary studies suggest that fibrinogen has relatively recently acquired its function as a clotting factor because many fibrinogen-related proteins in invertebrates have an important role in defense processes, such as pathogen recognition, agglutination, and bacterial lysis, however, not in clotting [2]. This applies also to other members of the coagulation cascade, as sequence homology analyses in vertebrates revealed that many clotting factors share ancestry with complement proteases [3]. Together these results show that the vertebrate coagulation system has developed from evolutionary related cascades involved in innate immunity [4]. It is therefore tempting to speculate that coagulation has a yet underestimated function in the host defense to infection. The coagulation cascade can be broken down into an extrinsic (tissue factor driven) and intrinsic pathway (contact activation). Both arms are initiated by limited proteolysis and are amplified in a snowball-like manner, eventually resulting in the generation of thrombin, which then initiates formation of a fibrin network [5].

The Gram-positive bacterium *Streptococcus pyogenes* is a major human pathogen that mainly causes local and self-limiting skin and throat infections. Infections can occasionally become invasive and develop into serious and life-threatening conditions such as streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis. Notably, both conditions are associated with high morbidity and mortality (for a review see [6]). The bacterium has evolved a variety of strategies to evoke activation of the coagulation cascade, involving for instance the induction of tissue factor on monocytes and endothelial cells by M proteins or an activation of the intrinsic pathway at the bacterial surface [7–9]. M proteins are streptococcal surface proteins and probably one of the best-known virulence determinants of this pathogen [10]. They can be released during infections [11] and act on monocytes to trigger cytokine induction and tissue factor up-regulation [8,12]. Recently we reported that soluble M protein triggers the release of pro-coagulant MVs from human peripheral blood mononuclear cells (PBMCs). Once released from PBMCs these MVs can initiate coagulation by activating both pathways in a sequential mode of action [13].

Author Summary

The coagulation system is much more than a passive bystander in our defense against exogenous microorganisms. Over the last years there has been a growing body of evidence pointing to an integral part of coagulation in innate immunity and a special focus has been on bacterial entrapment in a fibrin network. However, thus far, pro-coagulant MVs have not been discussed in this context, though it is known that their numbers can dramatically increase in many pathological conditions, including severe infectious diseases. In the present study we see a significant increase of pro-coagulant MVs in an invasive streptococcal mouse model, suggesting that their release is an immune response to the infection. We find that pro-coagulant MVs bind to *Streptococcus pyogenes* and promote clotting, entrapment, and killing of the bacteria in a fibrin network. As a proof of concept pro-coagulant MVs were applied as local treatment in the streptococcal infection model, and it was demonstrated that this led to a significantly improved survival in mice.

Apart from PBMCs MVs can be secreted from almost all other human blood-born cells, and depending on their cell activation MVs can differ in their composition and function. Elevated levels of MVs have been related to pathological conditions such as bleeding and thrombotic disorders, cardiovascular diseases, cancer, and infectious diseases [14]. They form sphere-shaped structures, less than 1 μm of diameter and limited by a lipid bilayer. In contrast to their cell of origin, MVs from activated cells expose negatively charged phospholipids, mainly phosphatidylserine (PS), on their outer membrane, which present a neo-exposed docking site for many plasma proteins including coagulation factors [15].

Despite an increasing knowledge on the role(s) of MVs in pathological processes e.g. as signaling molecules, in angiogenesis, and in initiation or propagation of coagulation and inflammation [14], their function in infectious diseases is only poorly understood. In the present study we investigated whether pro-coagulant MVs are part of the innate immune response by exposing antimicrobial activity. To this end we performed a number of *in vitro* and *in vivo* experiments to show that pro-coagulant MVs not only efficiently prevent the proliferation of *S. pyogenes* bacteria within a formed clot, but also that application of human MVs in a subcutaneous murine infection model dampens bacterial spreading and improves survival.

Results

Pro-coagulant MVs bind to *S. pyogenes*

PBMCs were isolated from human blood and stimulated with M1 protein as described in *Methods*. MVs were then purified as reported earlier [13] and the pro-coagulant activity of MVs was confirmed by measuring the clotting time (data not shown). For subsequent binding studies, pro-coagulant MVs were tagged with gold-labeled annexin V and incubated with *S. pyogenes* bacteria in the presence of 1% plasma. Figure 1A depicts transmission electron micrographs at lower and higher magnification. At higher magnification the figure shows that pro-coagulant MVs are bound to the bacterial surface in the presence of plasma.

To test whether the presence of MVs derived from other cells, interferes with the binding of pro-coagulant MVs from PBMCs, whole blood was stimulated with M1 protein. MVs were isolated and their binding to *S. pyogenes* was studied by transmission electron microscopy. Figure 1B (*upper panel*) shows that MVs isolated from M1 protein-activated blood bind to the bacterial surface. The origin of PBMC-derived MVs was confirmed by immunostaining with CD14, also showing that activation of blood with M1 protein caused an increase in binding of monocyte-derived MVs (Figure 1B, *middle panel*). To test whether the activation stage of the MVs contributes to binding, MVs were immunostained with an antibody against tissue factor. Figure 1B (*lower panel*) shows that only a few tissue factor-positive MVs were found attached to the bacteria, when MVs were isolated from non-stimulated blood. However, a more intensive antibody staining was recorded when MVs were recovered from M1 protein stimulated blood, showing that blood cell activation led to pro-coagulant MVs that bind to the bacterial surface. Based on these results we decided to use MVs isolated from PBMCs for all further experiments. MVs that were isolated from M1 protein stimulated PBMCs are therefore referred to as "pro-coagulant MVs" and from non-activated PBMCs as "ctrl. MVs" throughout the remaining part of this study.

The interaction of MVs with *S. pyogenes* was further investigated by fluorescence microscopy. Pro-coagulant or ctrl. MVs were labeled with PKH26 (red) and incubated with *S. pyogenes* in human plasma. After a 30 minute incubation step, aggregates of MVs and bacteria (DAPI-stained, blue) were observed (Figure S1), similar to those described by Timár and colleagues [16]. The number of MV-bacterial aggregates that exceeded 10 μm was quantified (Table 1). The data show that both types of MVs bind and aggregate bacteria, but incubation with pro-coagulant MVs induced more and larger aggregates when compared with ctrl-MVs (Table 1).

Clotting of *S. pyogenes* after opsonization with pro-coagulant MVs

Next we tested whether opsonization of *S. pyogenes* with pro-coagulant MVs, renders the bacteria susceptible for clotting. To this end, *S. pyogenes* bacteria were pre-incubated with pro-coagulant MVs in the presence or absence of human plasma, washed thoroughly to remove non-bound MVs, and added to recalcified plasma. Under these experimental settings clotting occurred within 162 s as shown in figure 2A. If, however, bacteria were incubated with pro-coagulant MVs in the absence of human plasma, no clotting was observed within 300 s and likewise, incubation of bacteria with plasma in the absence of pro-coagulant MVs prevented clotting (Figure 2A). Together the experiments imply that plasma protein(s) are required for the binding of pro-coagulant MVs to the bacteria and subsequent activation of clotting. Fibrinogen is a plausible candidate, as it is an abundant plasma protein and has high affinity for most streptococcal strains, including the AP1 strain, which was used in this study [9]. Therefore *S. pyogenes* bacteria were incubated with pro-coagulant MVs in the presence of normal or fibrinogen-depleted plasma, washed to remove non-bound MVs, and added to normal recalcified plasma. As before, when bacteria were pre-incubated with pro-coagulant MVs in the presence of normal plasma, clotting occurred within 169 s, while clotting was significant delayed (235 s) when bacteria were pre-incubated with pro-coagulant MVs in fibrinogen-depleted plasma, prior re-calcification with normal plasma (Figure 2B). Note that fibrinogen-depleted plasma was generated by defibrination and as fibrinogen was not completely removed (0,04 g/l are remaining), clotting was only delayed but not completely prevented.

Previous work has demonstrated that M1 protein from *S. pyogenes* is the main fibrinogen receptor on the AP1 strain used in this study [17]. To test whether M1 protein is also the major

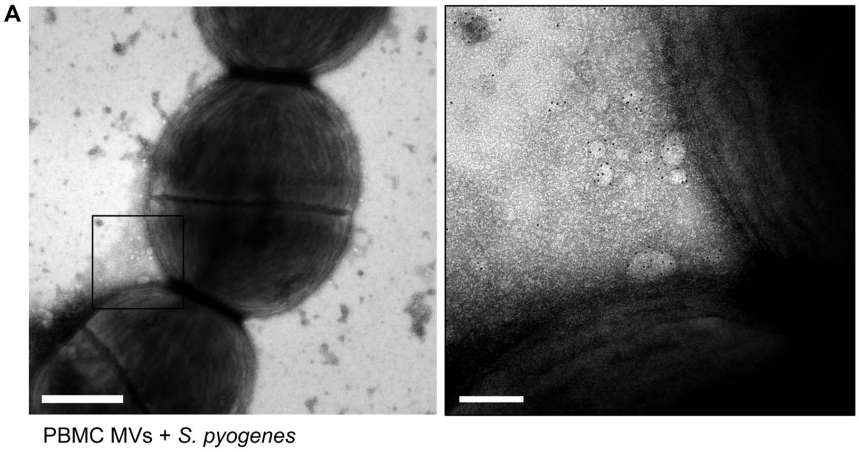
A
PBMC MVs + S. pyogenes
B
control
stimulated
overview
CD14
tissue factor
Blood MVs + S. pyogenes

Figure 1. MVs bind to *S. pyogenes*. **A**) Pro-coagulant MVs were gold-labeled with annexin V (5 nm) and incubated with *S. pyogenes* bacteria in the presence of 1% plasma. Samples were processed by negative staining and analyzed in a transmission electron microscope at two different magnifications. Arrows point to annexin V-positive MVs and scale bars represent 500 nm (left) or 100 nm (right). **B**) An overview of an *S. pyogenes* bacterium opsonized with ctrl. (*upper panel left site*) and pro-coagulant MVs (*upper panel right site*) is shown (Scale bar 500 nm). Ctrl. MVs (*left side*) and pro-coagulant MVs (*right side*) were immunostained with a gold-labeled antibody against CD14 (*middle panel*) or tissue factor (*lower panel*) and incubated with *S. pyogenes*. The scale bar indicates 100 nm.
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fibrinogen binding protein that mediates the interaction between bacteria and MVs, we employed an isogenic AP1 mutant strain (MC25), which does not express M1 protein on its surface [18]. Wildtype AP1 and MC25 bacteria were pre-incubated with pro-coagulant MVs in the presence of human plasma, washed thoroughly to remove non-bound MVs, and added to recalcified plasma. As depicted in figure 2C, MC25 bacteria tagged with pro-coagulant MVs were not as potent to induce clot formation as AP1 bacteria. The number of MV-bacterial aggregates was quantified by fluorescence microscopy and also in these experiments we found that the MC25 strain was not as effective as the AP1 strain to form aggregates (5 ± 2 *vs.* 49 ± 11) in plasma when opsonized with pro-coagulant MVs.

Finally we further investigated, whether other M proteins, either from the same serotype or from other serotypes, can recruit MVs to their surface. We therefore tested 14 clinical isolates, of which 5 were of the M1 type and 9 of other serotypes (Figure S2A and B). When subjecting these strains to clotting assays we found that all serotypes had similar pro-coagulant activities as seen for the AP1 strain.

Together the results show that the binding of pro-coagulant MVs to streptococci alters the bacterial surface from a non-coagulative to a pro-coagulative state. This interaction seems to be a common mechanism of group A streptococci, as also other serotypes explored similar clotting activities when incubated with pro-coagulant MVs. Moreover the data suggests that fibrinogen plays an important role in this chain of events.

Pro-coagulant MVs recruit, via fibrinogen, streptococcal adhesion factors to their surface

To study the role of fibrinogen as molecular bridge in more detail, surface plasmon resonance spectroscopy was employed. In a series of experiments we tested whether the activation state of MVs constitutes a regulatory mechanism that steers their affinity for fibrinogen. Sensor chips were coated with ctrl. or pro-coagulant MVs and probed with increasing concentration of fibrinogen. Though fibrinogen binding to both ctrl. MVs (Figure 3A) and pro-coagulant MVs (Figure 3B) was detected, determination of the association constants revealed that pro-coagulant MVs have a much higher affinity for fibrinogen than ctrl. MVs (0.019 nM vs. 3.3 μM, respectively) as shown in figure 3C.

The results from clotting experiments and fluorescent microscopy implicate an important role of M1 protein in binding

Table 1. Number of MV-bacteria aggregates (≥10 μm sized).

S. pyogenes + ctrl. MVs	*S. pyogenes* + pro-coagulant MVs
19±4	49±11

PKH-26 stained MVs and *S. pyogenes* were incubated in plasma for 30 min. 10 μl was dropped onto a cover slide, and counterstained with DAPI. Statistical analysis was carried out from 35 microscopic visual areas. Average and SEM are shown from 3 independent experiments.
doi:10.1371/journal.ppat.1003529.t001

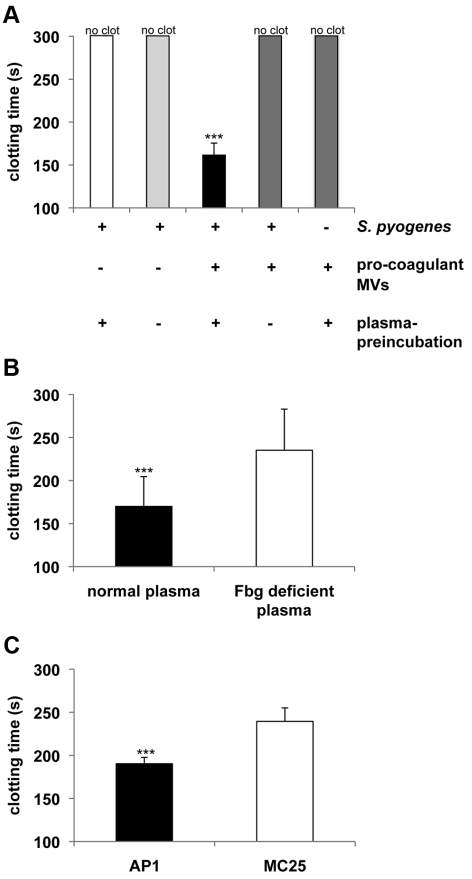
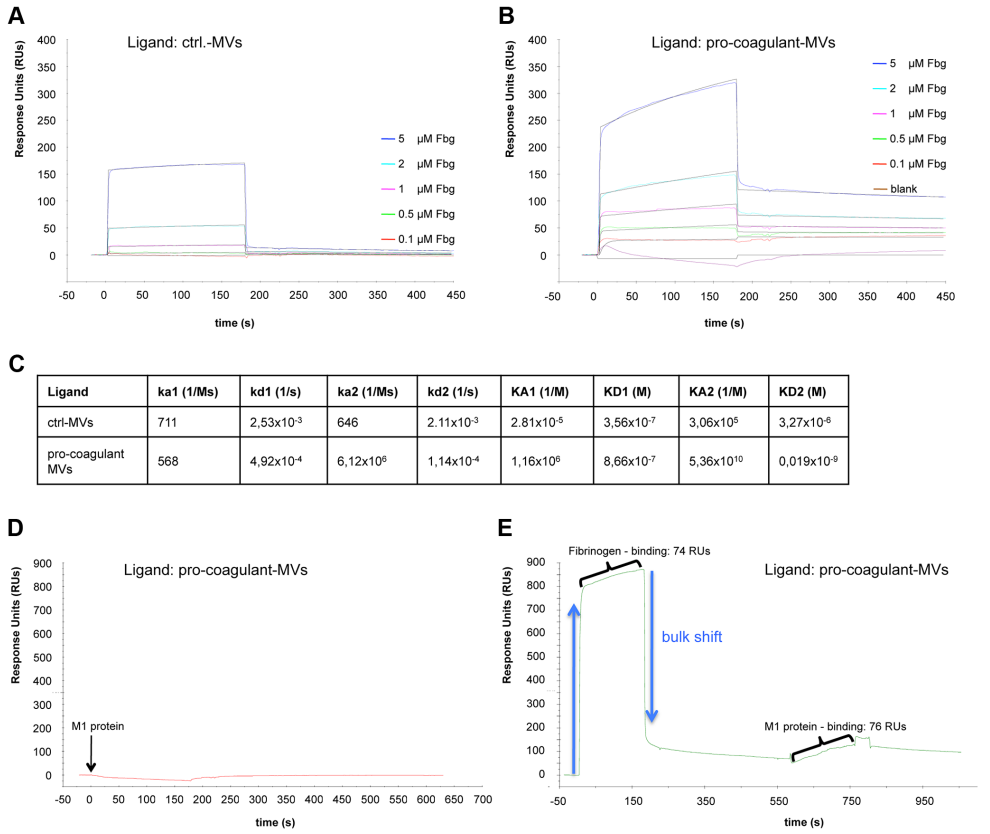


Figure 2. Pro-coagulant MVs bound to *S. pyogenes* induce clotting in human plasma. **A**) *S. pyogenes* were incubated with pro-coagulant MVs or buffer in the presence or absence of plasma for 30 min at 37°C. After washing, bacteria were added to recalcified plasma and clotting time was determined. **B**) *S. pyogenes* were incubated with pro-coagulant MVs in the presence of normal or fibrinogen (Fbg) depleted plasma for 30 min at 37°C. After washing, bacteria were added to recalcified plasma and clotting time was determined. **C**) Wildtype AP1 or MC25 bacteria were incubated with pro-coagulant MVs in the presence of plasma for 30 min at 37°C. After washing, bacteria were added to recalcified plasma and clotting time was determined. Clotting times were performed in triplicate. The data represent the means ± SD of 3 independent experiments. ***$P<0.001$.
doi:10.1371/journal.ppat.1003529.g002



Ligand	ka1 (1/Ms)	kd1 (1/s)	ka2 (1/Ms)	kd2 (1/s)	KA1 (1/M)	KD1 (M)	KA2 (1/M)	KD2 (M)
ctrl-MVs	711	$2{,}53\times10^{-3}$	646	2.11×10^{-3}	2.81×10^{-5}	$3{,}56\times10^{-7}$	$3{,}06\times10^{5}$	$3{,}27\times10^{-6}$
pro-coagulant MVs	568	$4{,}92\times10^{-4}$	$6{,}12\times10^{6}$	$1{,}14\times10^{-4}$	$1{,}16\times10^{6}$	$8{,}66\times10^{-7}$	$5{,}36\times10^{10}$	$0{,}019\times10^{-9}$

Figure 3. Binding properties of MV surfaces measured by SPR. Ctrl. MVs (**A**) or pro-coagulant MVs (**B**) were coupled to a sensor chip and subjected to injections with serial dilutions of fibrinogen (0.1–5 µM). Overlaid concentration-responses of the binding curves are shown. For both ligands, the data of the interaction with fibrinogen fit best to the heterogeneous binding model. The kinetic and affinity parameters are listed in (**C**). Pro-coagulant MVs were coupled to a sensor chip over which 100 µg/ml M1 protein was injected (**D**) or which was loaded sequentially, first with 5 µM fibrinogen, and then with 100 µg/ml M1 protein (**E**).
doi:10.1371/journal.ppat.1003529.g003

pro-coagulant MVs (see Figure 2C). To verify this conclusion we measured the interaction between M1 protein and pro-coagulant MVs, immobilized on a sensor chip, by surface plasmon resonance in the presence or absence of fibrinogen. Figures 3D+E illustrates that an interaction between M1 protein and the pro-coagulant MVs was only detectable when the chip was pre-incubated with fibrinogen, confirming fibrinogen's function as a bridging factor. In conclusion, the data show that MVs derived from activated cells expose additional binding sites for fibrinogen, which are required as docking sites for the streptococcal adhesion factor such as M1 protein or M proteins from other serotypes.

Fibrinogen-binding integrins are up-regulated on the surface of MVs

In order to investigate how pro-coagulant MVs can up-regulate additional fibrinogen binding-sites, mass spectrometry analysis was used, which allows the identification and quantification of intracellular, membrane associated, and secreted proteins of MVs. With this approach a total number of 169 proteins, with a false discovery rate of 1%, was identified in non-stimulated and pro-coagulant MVs (Table S1). In ctrl. MVs, 57% of the proteins were cytosolic, 23% secreted, 12% membrane-associated, and 8% mitochondrial origin (Figure S3). This composition changed drastically in pro-coagulant MVs, as here an increase in secreted and membrane associated proteins was found (36% and 28%, respectively), while a decrease in cytoplasm and mitochondrial proteins to 35% and 1% was measured (Figure S3). We also noted a rise in the concentration of 34 proteins recovered from pro-coagulant MVs comparing to ctrl. MVs (Table 2). In particular, leucocyte elastase levels were dramatically up-regulated (approximately 2500 times), but also higher levels of the fibrinogen-binding integrins CD18 (42 times) and CD11b (7.8 times) were

noted. Another integrin, alpha-V/beta-3, which is a receptor for a number of human proteins including fibronectin, laminin, and vitronectin were also found upregulated (2.9 times). Finally we noticed that proteins with antimicrobial functions such as lysozyme and neutrophil defensin 1 (3.7 times and 2.8 times, respectively) were also enriched in pro-coagulant MVs. Taken together the determination of the protein content in ctrl. and pro-coagulant MVs by mass spectrometry analysis revealed that, apart from two fibrinogen-binding integrins, other proteins with an important role in the early immune response, are also up-regulated in pro-coagulant MVs.

Table 2. Proteins from pro-coagulant MVs detected by mass spectometry analysis that were significant upregulated, compared to ctrl. MVs [37].

Proteins	*pro-coagulant MVs*
Platelet glycoprotein Ib	1,505907916
Zyxin	1,519533276
CD36 antigen	1,630243882
Integrin alpha 2b , alpha2bbeta3, vibronectin	1,774382368
Myeloperoxidase	1,858537826
guanine nucleotide binding protein (G protein),	2,086581862
LIM and senescent cell antigen-like domains 1	2,158272675
Intercellular adhesion molecule 3	2,592230491
Monocyte differentiation antigen CD14	2,646502683
Platelet glycoprotein IX	2,805388155
Neutrophil defensin 1	2,855469867
Leukosialin	2,888946866
Integrin beta chain, alphaV, beta3 (Vitronectin receptor)	2,891676956
HLA class I histocompatibility antigen	2,897177578
Cytochrome b-245	3,055074965
Solute carrier family 9	3,07933824
Leukocyte common antigen (CD45 antigen)	3,242725034
CD44 antigen	3,455159577
Lysozyme C	3,743112539
Ras-related protein	3,827823588
Tetraspanin-14	4,135484955
Annexin I	4,533846343
SH3 domain-binding glutamic acid-rich-like protein 3	4,659452596
Integrin alpha-M precursor (CD11b)	7,840017118
Sodium/potassium-transporting ATPase alpha-1 chain	8,213665434
Annexin V	11,14117886
Brain abundant, membrane attached signal protein 1	12,4888186
Annexin A2	16,74723805
Annexin VI	18,68650302
Alanyl (Membrane) aminopeptidase (CD13)	32,75718975
Integrin, beta 2 (CD18)	42,36002149
Human Cofilin	74,66172249
Ras-related protein Rap-2a	98,69200927
Leukocyte elastase	2449,565037

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Plasma clots induced by pro-coagulant MVs immobilize bacteria

Recent studies support the concept that clot formation at the site of infection entraps bacteria in the fibrin network, which in turn prevents bacterial spreading, and promotes bacterial elimination [19,20]. Based on these reports, we speculated that MVs could also act as a clotting initiator that chains the bacteria within a formed clot. To prove this hypothesis, *S. pyogenes* were incubated in recalcified plasma followed by the addition of ctrl. MVs or pro-coagulant MVs. Artificial phospholipids with pro-coagulant activity (PLs) or tissue factor (TF) containing samples served as positive controls. Stable clots were formed when pro-coagulant MVs, PLs, or tissue factor were added to the bacteria/plasma mixture, while loose and less compact clots were generated when the bacteria/plasma mixture was incubated with buffer or ctrl. MVs (not shown). The clot samples were covered with Tris-buffer containing 1% plasma and incubated for two or four hours at 37°C. Aliquots were collected from the supernatants and bacterial loads were determined. After two hours of incubation the number of released bacteria from plasma clots derived by pro-coagulant MVs was significantly decreased (9.7 times), when compared with the number found in the supernatants of samples incubated with ctrl. MVs (Figure 4A). After the four-hour incubation, samples treated with buffer of ctrl. MVs contained high loads of streptococci. As seen before, incubation of bacteria in a plasma clot derived from pro-coagulant MVs prevented the escape of bacteria from the clots (more than 12 times, comparing to ctrl-MVs) and also PLs or tissue factor induced clots had a similar effect (Figure 4B). These data demonstrate that bacteria are efficiently trapped and immobilized if they are opsonized with pro-coagulant MVs.

Plasma clots induced by pro-coagulant MVs have antibacterial activity

It has recently been shown that activation of the coagulation cascade on the surface of *S. pyogenes* leads to an induction of antimicrobial activity [20]. To investigate whether antimicrobial activity is also seen when clotting is induced by pro-coagulant MVs, additional bacterial growth experiments were performed. Streptococci were mixed with plasma and clotting was initiated by adding pro-coagulant MVs, PLs, or tissue factor. Ctrl. MVs or buffer served as controls. After 30 min, clots were homogenized and bacterial loads determined. As seen in figure 5A, bacterial counts were significant reduced to 20–30% in samples treated with pro-coagulant MVs, PLs, or tissue factor, when compared with ctrl. MVs. Samples incubated with buffer only, served as a control (100% growth). Clot formation appears to be the critical moment in these experiments, since no reduction in bacterial growth was monitored when calcium was omitted and thus clotting prevented (Figure 5B). Similar results, though not a complete reversion, were seen when recalcified samples were treated with a peptide (Gly-Pro-Arg-Pro) that prevents the polymerization of fibrin monomers (Figure 5C) [21].

To visualize the bacteria, samples were subjected to scanning electron microscopy (Figure 5D–F). In the absence of pro-coagulant MVs, *S. pyogenes* bacteria appear as intact cocci when incubated for 30 min in plasma (Figure 5D). In the next series of experiments, pro-coagulant MVs (Figure 5E) were added to the plasma bacteria mixture. Figure 5F illustrates that after activation with pro-coagulant MVs, bacteria were weaved in a fibrin network. It also appears that the morphology of bacteria was not significant compromised, as the bacterial cell membrane seems to be still intact (Figure 5F). These images may indicate that the

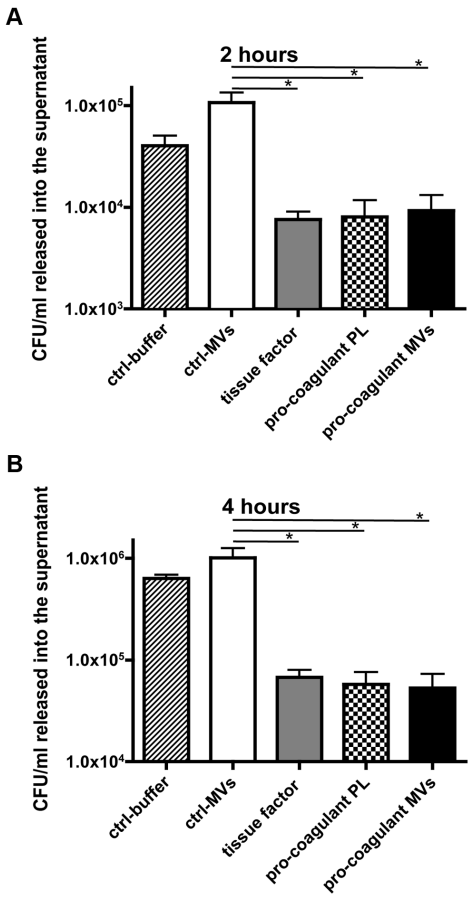

Figure 4. Pro-coagulant MV-derived clots prevent bacterial dissemination. Recalcified plasma was incubated with 2×10^5 CFU/ml *S. pyogenes* and clot formation was triggered with pro-coagulant PLs or MVs or 2 pM tissue factor. Tris-buffer (ctrl-buffer) or ctrl-MVs samples were used as negative controls. The clots were covered with 1% plasma, incubated at 37°C and supernatants were plated after 2 (**A**) and 4 hours (**B**). The data represent the means ± SD of 3 independent experiments, *P<0.05.
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effect seen is of bacteriostatic nature rather than bactericidal, however, more experimental support is needed to prove this conclusion. Taken together the data suggest that pro-coagulant MVs are able to prevent bacterial spreading and impair bacterial proliferation inside a plasma clot.

Pro-coagulant MVs are increased in plasma samples from mice infected with *S. pyogenes*

Recently we reported that pro-coagulant MVs from patients suffering from streptococcal sepsis are significant increased [13]. To test whether this can also be observed in an invasive animal model of streptococcal infection, mice were subcutaneously infected. The animals were sacrificed after three time points (10, 24–30, and 42–48 hours after infection) and plasma samples were recovered by cardiac puncture. Figure 6A depicts that the TF content in the plasma samples was not significant raised 10 hours after infection, but was significantly increased at the later time points (Figure 6A). Similar results were seen when measuring the concentrations of pro-coagulant MVs, though they already start to peak 10 hours after infection (Figure 6B). Thus, the data show that the generation of pro-coagulant MVs is part of the host response to invasive infection with *S. pyogenes*.

Local treatment with pro-coagulant MVs dampens systemic bacterial spreading and improves survival in infected mice

The role of MVs in systemic infectious diseases is currently not completely understood, but it has been speculated that elevated levels in the early phase of sepsis may have protective effects [22]. We therefore studied whether the local application of pro-coagulant MVs to the site of infection may improve the outcome of the disease. Three groups of mice were infected with *S. pyogenes* bacteria and were treated either with vehicle, ctrl. MVs or pro-coagulant MVs. While application of ctrl. MVs failed to improve survival as compared to control (vehicle), treatment with pro-coagulant MVs significant prolonged survival time and decreased the mortality rate (Figure 7A).

The subcutaneous injection of pro-coagulant MVs also had an impact on the bacterial load in different organs of the infected mice. Mice received a subcutaneous injection of *S. pyogenes* bacteria and simultaneously a single dose of pro-coagulant MVs. Infected animals were sacrificed 18 hours after infection, and bacterial loads in the blood, liver, and spleen were determined. As depicted in figure 7B–D, treatment with pro-coagulant MVs resulted in decreased numbers of bacteria in all organs when compared with non-treated animals. These results are in line with previous conclusions and may indicate that pro-coagulant MVs are part of the early host defense to an infection at an early stage of the infectious disease progression.

Discussion

Pro-coagulant MVs constitute one of the main reservoirs of blood-borne TF, which are released from monocytes, macrophages, or endothelial cells with inducible TF expression [23] and they are therefore considered to be key determinants of the hemostasis equilibration [24]. Notably, the number of pro-coagulant MVs can significantly increase in patients suffering from sepsis as reported by us and other groups [13,25]. These findings raise the question whether they are part of the host response to infection or rather contribute to systemic hemorrhagic complications, such as disseminated intravascular coagulation (DIC) in severely ill patients. Reid and Webster recently published a review article on the role of MVs in sepsis [22]. The authors conclude that MVs are beneficial at the early stage of sepsis as they can compensate for some of the host's systemic reactions [22]. Our findings support this notion, because local treatment with pro-coagulant MVs significantly prevents bacterial dissemination and improves survival. Moreover, activation of PMBCs is triggered by the binding of M1 protein to toll-like receptor 2 [12], which suggests that formation and release of pro-coagulant MVs follows the principles of pattern recognition and are therefore part of the innate immune reaction. However, as seen for many other host defense mechanisms, the systemic induction of pro-coagulant MVs may contribute to severe complications, such as DIC. A better

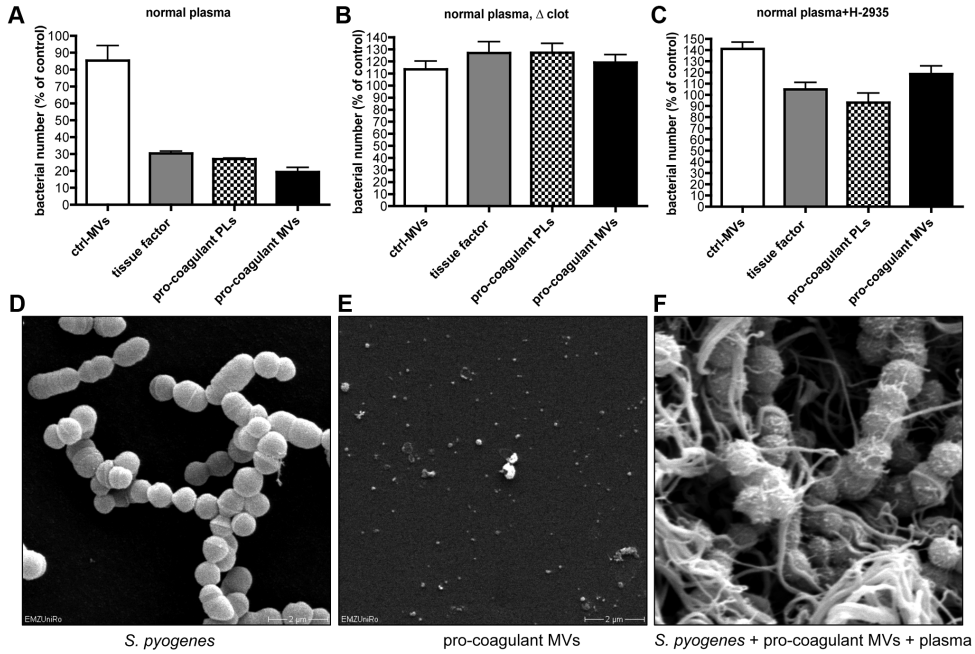

Figure 5. Determination of the antimicrobial activity of plasma clots induced by pro-coagulant MVs. A) Recalcified plasma was incubated with 2×10^5 CFU/ml *S. pyogenes* in the presence of ctrl. MVs, pro-coagulant MVs, PLs, or 2 pM tissue factor. Tris-buffer was used as a control (100%). **B**) Samples were prepared as described in A, but clot formation was prevented by omitting calcium, or the addition of 1.5 mg/ml Gly-Pro-Arg-Pro (**C**). Scanning electron microscopy of *S. pyogenes* (**D**), pro-coagulant MVs (**E**), and a plasma clot with *S. pyogenes* that was induced by the addition of pro-coagulant MVs (**F**). The data represent the means ± SD of 3 independent experiments.
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understanding of the molecular mechanisms that modulates the tightly regulated process may lead to the development of novel antimicrobial therapies with different modes of action that can be used for local treatment or in systemic complications.

Microvascular thrombosis and the formation of a fibrin network can be considered as an efficient and early response of the host defense against bacteria that can lead to an immobilization of bacteria and thereby attenuates the spreading of the pathogen [19,20,26]. Our studies show that pro-coagulant MVs bind to *S. pyogenes* and that this interaction leads to an alteration of the bacterial surface into a pro-coagulative state. We found that fibrinogen is a docking molecule that attaches protein M1, a streptococcal surface-bound adhesion factor, to pro-coagulant MVs. Subsequent mass spectroscopic analysis revealed an up-regulation of the fibrinogen binding integrins (CD18 and CD11b, respectively) at the surface of pro-coagulant MVs. This chain of events presents a plausible explanation as to how pro-coagulative MVs achieve their affinity for *S. pyogenes*. However, it cannot be ruled out that other proteins such as fibronectin, vitronectin or laminin are also involved [27–29]. Notably, many of these host adhesion factors are also receptors for other bacterial pathogens [30]. Thus their binding to pro-coagulant MVs may represent some kind of pattern recognition mechanism that allows the targeting of other microorganisms in a more general sense. Future work will show whether pro-coagulant MVs are also interacting with other bacterial species and whether this involves the recruitment of fibrinogen and/or other host adhesion proteins.

Our results show that plasma clots that were induced by pro-coagulant MVs can immobilize *S. pyogenes* as efficiently as clots induced by tissue factor or artificial phospholipids. Importantly, clots formed in the presence of pro-coagulant MVs had antimicrobial activity against *S. pyogenes*, which could be explained by their cargo containing antimicrobial peptides and proteins. However, we noted that clots formed by the addition of tissue factor or artificial phospholipids, were also able to kill the entrapped bacteria. It therefore remains to be determined as to what extent the peptides/proteins with antimicrobial activity from pro-coagulant MVs contribute to bacterial killing, or if there are bactericidal substances generated during the activation of the coagulation cascade. The latter hypothesis is supported by recent findings that many coagulation factors contain a sequence at their carboxy-terminal part with an antimicrobial activity [31,32]. Taken together, our data show that activation of the coagulation cascade and the formation of a fibrin network are important mechanisms to prevent bacterial dissemination and proliferation. As pro-coagulant MVs are induced at an early stage during bacterial infection, their local interaction with bacteria can be considered as part of the early immune response.

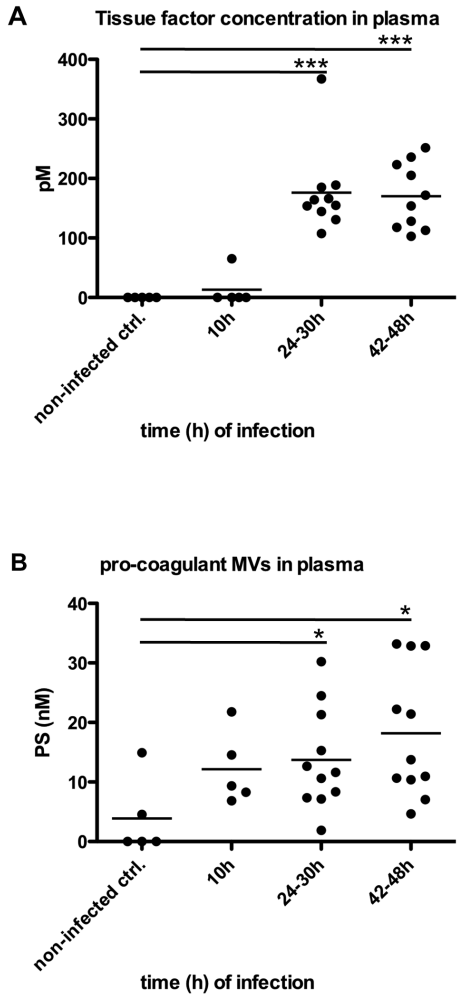

Figure 6. Analysis of plasma from septic mice. Mice were subcutaneously infected with 2×10^7 CFU *S. pyogenes* bacteria and plasma samples were collected at 0, 24, 30, 42 and 48 hours after infection (n = 5–11/group). Tissue factor activity (**A**) and pro-coagulant MVs (**B**) expressed as PS equivalent (nM) were measured. *P<0.05, ***P<0.0001.
doi:10.1371/journal.ppat.1003529.g006

Material and Methods

Bacterial strains and culture conditions

The *S. pyogenes* strain AP1 (40/58) serotype M1 and its M1-derivate MC25 have been described previously [18,33]. All other *S. pyogenes* strains were clinical isolates from our strain collection that have been characterized by standard microbiological procedures. Bacteria were grown overnight in Todd-Hewitt broth (THB; GIBCO) at 37°C and 5% CO_2.

Material

M1 protein was purified from the supernatant of *S. pyogenes* MC25, as previously described [18]. Artificial pro-coagulant phospholipids were from Rossix (Sweden). Recombinant tissue factor and anti TF were from American Diagnostica (Germany). Anti CD14 was from Dako (Denmark).

Preparation of MVs from peripheral blood mononuclear cells (PBMCs)

PBMC isolation, stimulation as well as MV purification were performed as described previously and used at concentration range from 50 to 150 MPs/μl [13].

Clotting experiments

S. pyogenes bacteria from 10 ml overnight culture were washed and resuspended in 1 ml 10 mM HEPES-buffer (2×10^9 CFU/ml). 150 μl bacteria and 30 μl MVs - in the presence or absence of 300 μl human plasma – were mixed and incubated for 30 min. at 37°C. Alternatively, fibrinogen depleted plasma (Affinity Biologicals, Canada) was used. Bacteria were washed 3 times with HEPES-buffer by centrifugation (1550× g for 10 min.), and finally resolved in HEPES-buffer. Clotting time was measured in a coagulometer (Amelung) after addition of reaction mixtures to recalcified normal human plasma.

Bacterial immobilization in plasma clots

100 μl recalcified normal plasma was mixed with 25 μl 2×10^5 CFU *S. pyogenes* bacteria, 25 μl MVs or pro-coagulant PLs (0.25 mM, Rossix), or 2 pM tissue factor (American Diagnostica) and incubated at 37°C for 5 min. The clots were covered with 10 mM Tris-buffer containing 1% plasma. At the indicated time points, 100 μl aliquots of the supernatant 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.

Antimicrobial activity of plasma clots

Plasma clots were produced as described above, covered with Tris-buffer containing 1% plasma and incubated at 37°C for 30 min. Alternatively, the tetrapeptide Gly-Pro-Arg-Pro (Bachem) was added to prevent clotting (1.5 mg/mL final concentration). After incubation clots were disrupted in a Ribolyser (Hybaid, 30 sec at speed 4.0) and the homogenate was plated directly onto blood agar. The number of bacteria was determined by counting colonies after 18 hours of incubation at 37°C.

Fluorescence microscopy

MVs were labeled with the red fluorescence aliphatic chromophore PKH26 dye (Sigma), which intercalate into lipid bilayers [34]. After labeling, MVs were washed and centrifuged as described [13]. 150 μl bacteria (2×10^9 CFU/ml) and 30 μl labeled MVs were mixed in 300 μl human plasma and incubated for 30 min at 37°C. After incubation 10 μl of the mix was dropped onto a cover slide, counterstained with DAPI (Invitrogen) and visualised by a BX60 fluorescence microscope and 100×1.3 or 60×1.25 UplanFl objectives (Olympus, Hamburg, Germany).

Negative staining and transmission electron microscopy

Human proteins (annexin V, anti CD14 AB, and anti TF AB) were labeled with colloidal gold (15 and 5 nm in diameter, BBI International) as described earlier [35]. MV/*S. pyogenes* preparations were mixed with gold-labeled 20 nM proteins for 20 min at room temperature and processed for negative staining [36].

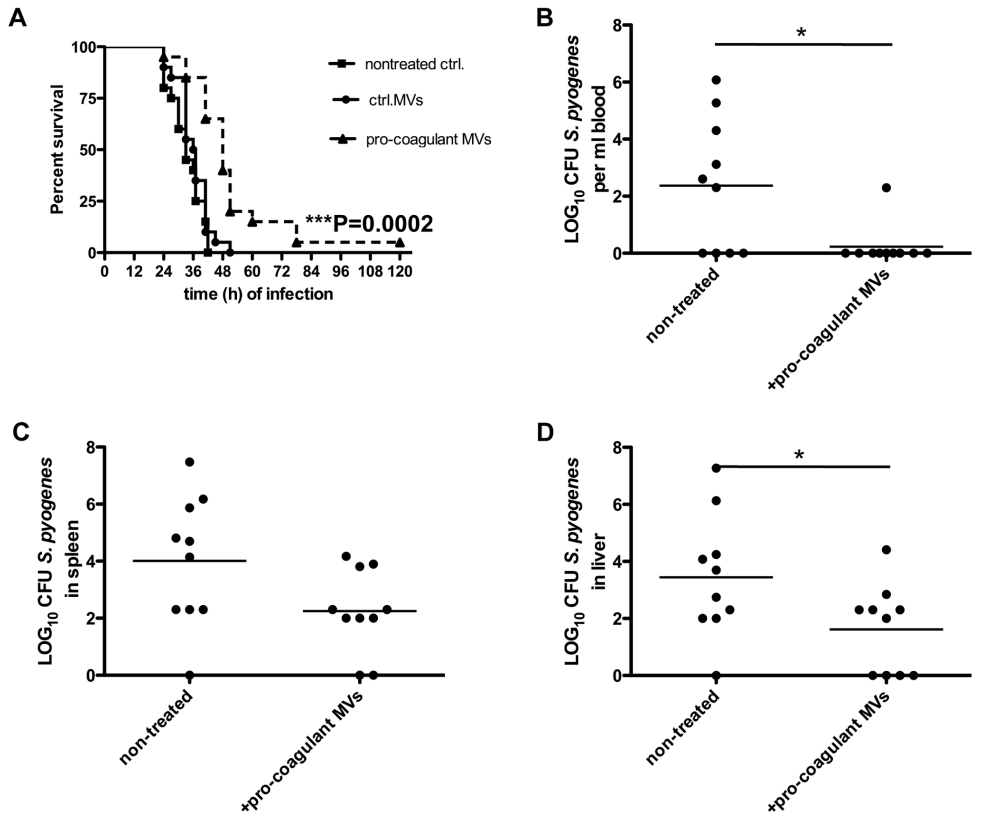

Figure 7. Treatment with pro-coagulant MVs in a mouse model of *S. pyogenes* sepsis dampens bacterial dissemination and improves survival. A) Mice were subcutaneously infected with 2×10^7 CFU *S. pyogenes* bacteria, immediately treated with 100 µl ctrl. or pro-coagulant MVs (150 MPs/µl, derived from 2×10^6 PBMCs) and survival was monitored up to 120 h after infection. Results show 4 independent experiments with MVs from 4 different donors and a total amount of 20 animals per group. (**B–D**) Mice were infected as described above and were immediately treated with 100 µl pro-coagulant MVs (150 MPs/µl, derived from 2×10^6 PBMCs), non-treated mice served as controls. Eighteen hours after infection, mice were killed and bacterial loads in the blood (**B**), spleen (**C**), and liver (**D**) were determined. Data are presented as means of 10 mice per group and were obtained from 2 independent experiments (*$P<0.05$).
doi:10.1371/journal.ppat.1003529.g007

Scanning Electron Microscopy (SEM)

Clots were fixed with 2.5% glutaraldehyde overnight. Samples were washed 2–3 times with 0.1 M sodium phosphate buffer (pH 7.3), dehydrated with a series of increasing ethanol concentrations (5 minutes in 30%, 5 minutes in 50%, 10 minutes in 70%, 10 minutes in 90% and two times 10 minutes in ethanol absolute), and dried with CO_2 by critical point method with a Emitech dryer as outlined by the manufacturer. Dried samples were covered with gold to a 10 nm layer and scanned with a Zeiss DSM 960A electron microscope.

TF activity ELISA

The Actichrome TF activity assay kit (American Diagnostica) was used to quantify the TF pro-coagulant activity in the plasma samples [13].

Measurement of MP pro-coagulant activity

The Coa-MP activity kit (Coachrom Diagnostica) was used according to the instructions of the manufactory, to measure the pro-coagulant activity of MVs in plasma [13].

Proteomic analysis

Protein digestion was carried out as previously described [37]. The resulting peptide mixtures were concentrated using spin-columns from Harvard Apparatus using the manufactures' instructions.

The hybrid Orbitrap-LTQ XL mass spectrometer (Thermo Electron, Bremen, Germany) was coupled online to a split-less Eksigent 2D NanoLC system (Eksigent technologies, Dublin, CA, USA). Peptides were loaded with a constant flow rate of 10 µl/min onto a pre-column (Zorbax 300SB-C18 5×0.3 mm, 5 µm, Agilent

technologies, Wilmington, DE, USA) and subsequently separated on a RP-LC analytical column (Zorbax 300SB-C18 150 mm×75 µm, 3.5 µm, Agilent technologies) with a flow rate of 350 nl/min. The peptides were eluted with a linear gradient from 95% solvent A (0.1% formic acid in water) and 5% solvent B (0.1% formic acid in acetonitrile) to 40% solvent B over 55 minutes. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS (from m/z 400 to 2000) and LTQ-MS/MS acquisition. Four MS/MS spectra were acquired in the linear ion trap per each FT-MS scan which was acquired at 60,000 FWHM nominal resolution settings using the lock mass option (m/z 445.120025) for internal calibration. The dynamic exclusion list was restricted to 500 entries using a repeat count of two with a repeat duration of 20 seconds and with a maximum retention period of 120 seconds. Precursor ion charge state screening was enabled to select for ions with at least two charges and rejecting ions with undetermined charge state. The normalized collision energy was set to 30%, and one microscan was acquired for each spectrum.

The data analysis was performed as previously described [37]. Briefly, the MS2 spectra were searched through the X! Tandem 2008-05-26 search engine [38] against the human protein database. The search was performed with semi-tryptic cleavage, specificity, 1 missed cleavages, mass tolerance of 25 ppm for the precursor ions and 0.5 Da for fragment ions, methionine oxidation as variable modification and cysteine carbamidomethylation as fixed modification. The database search results were further processed using the Trans-Proteomic pipeline, version 4.4.0 [39].

Surface plasmon resonance binding study

Real time biomolecular interaction was analyzed with a BIAcore3000 system (Biosensor, La Jolla, CA) using L1 sensor chips [40]. The L1 sensor chip comprises a carboxymethyl dextran hydrogel derivatized with lipophilic alkyl chain anchors to capture phospholipid vesicles. Experiments were performed at 25°C with 10 mM TRIS, 0.9% NaCl, pH 7.4 as running buffer and PBS (pH 7.4) as immobilization buffer. MVs were coated onto the L1-sensor chip according to the manufacturer' s instructions. Briefly, the L1 chip surface was washed by 2×3 minute injections of 40 mM N-octyl-β-D-glucopyranoside (Roth, Germany) at a flow-rate of 10 µl/min. MVs in PBS were then injected over the sensor for 30 min at a flow-rate of 2 µl/min resulting in 2000–2200 response units (RUs) of ctrl or pro-coagulant MVs. To remove residual multilayer structures and loosely bound vesicles, a short pulse of 10 mM NaOH was applied. Subsequently, BSA (0.1 mg/ml, 5 min) was added to block non-specific surface binding. The resulting bilayer linked to the chip surface was taken as a model MV-membrane surface for studying fibrinogen binding. Pure buffer solutions and the solution containing fibrinogen or M1 protein were applied at a flow rate of 10 µl/min. Following each cycle of analysis, the sensorchip was regenerated either with short pulses of 10 mM NaOH leaving the MV-lipid monolayer intact for additional interaction studies, or with 40 mM N-octyl-β-D-glucopyranoside stripping the MV-lipid layer from the surface in order to adsorb new MVs.

SPR data analysis

The sensorgrams for each fibrinogen–MV bilayer interaction were analyzed by curve-fitting. Data from 5 concentrations were selected for statistic analysis. RUs of the running buffer were subtracted from the RUs of the sample solution. The data were analyzed using the Biaevaluation 3.0 software (Biacore) that offers various reaction models to perform complete kinetic analyses. The data from the BIAcore sensorgrams were fitted globally, and the heterogeneous ligand model resulted in optimum mathematical fits, reflected by low χ^2 values (<5).

Animal experiments

The subcutaneous infection model with *S. pyogenes* AP1 strain were performed in female Balb/C mice as described previously [33]. This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments the *Landesveterinär- und Lebensmitteluntersuchungsamt Rostock* (Permit Number: *7221.3-1.1-031/10.*).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, Version 4.00. The P-value was determined by using the unpaired t-test (comparison of 2 groups) or the log-rank test (comparison of survival curves). All samples were analyzed in triplicate and all experiments were performed at least three times, if not otherwise declared. The bars in the figures indicate standard deviation.

Supporting Information

Figure S1 Representative transmission fluorescence microscopy images (from 3 experiments) from *S. pyogenes* incubated with MVs in plasma. 150 µl bacteria (2×10^9 CFU/ml, blue) and 30 µl PKH-26 labeled MVs (red) were mixed in 300 µl human plasma and incubated for 30 min at 37°C. After incubation 10 µl of the mix were dropped on a coverslide, counterstained with DAPI (blue, Invitrogen) and investigated. Scale bars represent 10 µm.
(TIF)

Figure S2 Clotting of different M1 (A) or M protein (B) *S. pyogenes* strains after incubation with pro-coagulant MVs. Bacteria were incubated with pro-coagulant MVs in the presence of plasma for 30 min at 37°C. After washing, bacteria or buffer (buffer-ctrl.) were added to recalcified plasma and clotting time was determined. Clotting times were performed in triplicate. The data represent the means ± SD of 2 independent experiments.
(TIF)

Figure S3 Subcellular location of proteins from PBMC derived MVs identified by hybrid orbitrap mass spectrometry analysis.
(TIF)

Table S1 Result of proteomic analysis of ctrl-MVs and pro-coagulant MVs from PBMCs. All identified proteins are listed, with a false discovery rate of 1%.
(XLSX)

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Author Contributions

Conceived and designed the experiments: SO JM BK HH. Performed the experiments: SO JW AIO. Analyzed the data: SO JM. Contributed reagents/materials/analysis tools: AIO MM JM. Wrote the paper: SO HH.

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