Bacterial pathogens activate plasminogen to breach tissue barriers and escape from innate immunity

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**Bacterial pathogens activate plasminogen to breach tissue barriers and escape from
innate immunity**

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Key terms

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Abstract

Both coagulation and fibrinolysis are tightly connected with the innate immune system. Infection and inflammation cause profound alterations in the otherwise well-controlled balance between coagulation and fibrinolysis. Many pathogenic bacteria directly exploit the host's hemostatic system to increase their virulence. Here, we review the capacity of bacteria to activate plasminogen. The resulting proteolytic activity allows them to breach tissue barriers and evade innate immune defense, thus promoting bacterial spreading.

Y. pestis, streptococci of group A, C and G and *S. aureus* produce a specific bacterial plasminogen activator. Moreover, surface plasminogen receptors play an established role in pneumococcal, borrelial and group B streptococcal infections.

This review summarizes the mechanisms of bacterial activation of host plasminogen and the role of the fibrinolytic system in infections caused by these pathogens.

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Introduction

The coagulation system and the innate immune defense share the same evolutionary origin. Tissue damage, as well as bacterial infection and local inflammation trigger activation of the coagulation system, which results in thrombin generation and fibrin deposition. Fibrin not only seals off leaking blood vessels, but also constitutes a first barrier against bacterial spread. Moreover, it provides a scaffold for inflammatory cells that aim at remodeling and restoring normal tissue architecture. The coagulation cascade is balanced by an anticoagulant system that avoids excessive coagulation and a fibrinolytic system that is able to dissolve fibrin clots. Excessive activation of both the coagulation and the fibrinolytic system contributes to the mortality of severe infections and inflammatory diseases (Verhamme & Hoylaerts, 2009).

Plasmin, the central enzyme of the fibrinolytic system, is a serine protease that is derived from its inactive zymogen precursor, plasminogen (Plg). Although fibrin is considered to be the main physiological substrate of plasmin, the proteolytic spectrum of plasmin also includes extracellular matrix (ECM) proteins. Besides fibrinolysis, Plg is implicated in many physiological processes such as cell migration, wound healing, angiogenesis and embryogenesis. Malignant cells can usurp the fibrinolytic system to invade tissues and disseminate to other body sites (Law et al., 2013).

Bacterial pathogens have developed various ways to interact with Plg and use the host's powerful proteolytic system to their own advantage. Abusing these otherwise tightly controlled cascades allows them to escape primary defense systems and to promote their

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3 dissemination through tissue barriers. While a few bacteria produce specific Plg activators,
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5 of which streptokinase, staphylokinase, and the Pla protease from *Y. pestis* are the most
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7 renowned, a vast group of pathogens can capture the host's Plg to their surface. In this
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9 review, we discuss the role of coagulation and fibrinolysis in inflammation, and focus on Plg
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11 activation as a virulence strategy in bacterial infections.
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The fibrinolytic system: a tightly controlled, broad-spectrum proteolytic system

The central component of the fibrinolytic system is plasminogen (Plg), the zymogen form of plasmin. Plasmin cleaves insoluble fibrin polymers into soluble fragments (Law et al., 2013, Rijken & Lijnen, 2009). Similar to other protease-dependent cascade systems, such as the coagulation and the complement system, its activity is regulated by different activators and inhibitors (**Figure 1**).

Plasminogen (Figure 2)

Plasminogen is a 90 kDa proenzyme that is abundantly present in plasma with concentrations ranging from 180 to 200 µg/ml or about 2 µM. Conversion to active plasmin, by cleavage of the peptide bond Arg⁵⁶¹-Val⁵⁶² by specific Plg activators leads to the formation of a heavy chain and a light chain that remain joined through two disulfide bridges (Rijken & Lijnen, 2009) (**Figure 2**). While the light chain contains the catalytic triad His⁶⁰³, Asp⁶⁴³ and Ser⁷⁴¹, which forms the serine protease active site, the heavy chain consists of five kringle domains with lysine-binding sites that allow interaction with target molecules and Plg receptors (PlgRs) (Castellino & Ploplis, 2005, Law et al., 2013). Binding to PlgRs facilitates conversion to plasmin and receptor-bound plasmin is protected from inactivation by the physiological inhibitor α₂-antiplasmin. The plasmin-mediated removal of an N-terminal 8 kDa activation peptide turns native Plg (Glu-Plg) into Lys-Plg, with a more open conformation allowing stronger interaction with target molecules and also facilitating conversion to plasmin (Castellino & Ploplis, 2005, Law et al., 2013).

Plasminogen activators

Mammals have two distinct Plg activators, tissue-type plasminogen activator (t-PA) and urokinase (u-PA) (Rijken & Lijnen, 2009). The 70 kDa t-PA is produced mainly by endothelial cells and activates Plg in the presence of fibrin (Lijnen & Collen, 1988), leading to lysis of intravascular fibrin clots. u-PA (54 kDa) binds to cellular u-PA receptors (u-PAR) and activates Plg at the cell surface (Blasi et al., 1986, Vassalli et al., 1985). This cell-surface associated plasmin activity is involved in cell adhesion and cell migration through ECM (Chapman, 1997, Law et al., 2013, Miles & Parmer, 2013, Rijken & Lijnen, 2009).

Although these Plg activators regulate very distinct processes, t-PA- or u-PA-deficient mice have only minor changes in their fibrinolytic capacity. Only with combined t-PA- and u-PA-deficiency extensive spontaneous fibrin deposition will develop, suggesting that the roles of t-PA and u-PA are in part redundant (Bugge et al., 1996a, Carmeliet et al., 1994).

Plasmin substrates and physiological functions of plasmin

Plasmin is a serine protease that cleaves peptide bonds C-terminal of a lysine or arginine residue. Plasmin has a broad spectrum of substrates. Fibrin is considered as the main physiological substrate of plasmin, but plasmin also degrades ECM proteins such as laminin, fibronectin, vitronectin and heparan sulfate proteoglycans. Although collagen is not susceptible to plasmin digestion, plasmin can activate procollagenases, indirectly leading to collagen degradation (Makowski & Ramsby, 1998, Monea et al., 2002, Murphy et al., 1992).

The ECM degradation resulting from this crosstalk between proteolytic enzyme systems is involved in the migration of leukocytes to the site of inflammation (Del Rosso et al., 2008).

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Malignant cells also rely on the Plg system for tumor cell invasion and u-PA is linked to metastatic properties of cancer cells (Laufs et al., 2006).

Plasmin inhibition

To avoid widespread proteolytic tissue degradation by plasmin, its activity is controlled not only at the level of Plg activation, but also by direct inactivation of plasmin. In addition, PlgRs confine the activity of plasmin to the cell surface, thus protecting against diffuse proteolytic damage (Miles & Parmer, 2013, Rijken & Lijnen, 2009).

Although the aspecific protease inhibitor α_2 -macroglobulin, abundantly present in plasma, inactivates plasmin, the main physiological plasmin inhibitor is the serine protease inhibitor (serpin) α_2 -antiplasmin (Rijken & Lijnen, 2009). α_2 -antiplasmin inactivates plasmin by covalently binding the serine residue of the catalytic site. This process is facilitated by the interaction between the kringle domains 1-3 of plasmin and the carboxy-terminal domain of α_2 -antiplasmin. Fibrin and PlgRs both use the lysine-binding sites to bind plasmin(ogen), which explains why fibrin and cell-surface bound plasmin is less susceptible to α_2 -antiplasmin inactivation (Rijken & Lijnen, 2009).

The plasminogen activator inhibitors (PAIs) also belong to the serpin family. PAI-1 inhibits t-PA and u-PA, and is implicated in cell migration, tumor development and cardiovascular diseases (Declerck & Gils, 2013, Lijnen, 2005). The exact role of the mainly intracellularly located PAI-2 as inhibitor of fibrinolysis is more controversial (Gardiner & Medcalf, 2014).

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a carboxypeptidase. The removal of C-terminal lysine residues of fibrin and PlgRs by TAFI impairs Plg binding. TAFI is activated by the thrombin-thrombomodulin complex and links coagulation, inflammation and fibrinolysis (Colucci & Semeraro, 2012, Rijken & Lijnen, 2009).

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The role of fibrin and fibrinolysis in host defense

Coagulation as well as fibrinolysis are closely intertwined with the innate immunity. A better understanding of the complex interactions between the hemostatic and the immune system is crucial to comprehend the manipulation of these pathways by bacteria.

Fibrin and host defense: coagulation as part of the innate immunity

The extensive crosstalk between coagulation and inflammation is reviewed in detail elsewhere and is beyond the scope of this article (Levi & Van Der Poll, 2010). Both systems, directed to protect the host from physical or biological injury, share a common evolutionary origin. An example is the clotting of hemolymph in the horseshoe crab as a response to either exoskeleton damage or bacterial infection (Delvaeye & Conway, 2009).

It has long been known that fibrin deposition is essential for abscess formation, allowing the host to encapsulate and prevent further spread of bacterial infections (Luo et al., 2013). Fibrinogen depletion leads to impaired abscess formation in mice after intraperitoneal inoculation, and causes more widespread disease (McRitchie et al., 1991). Mice with reduced fibrinogen levels or reduced thrombin activity are markedly more sensitive to infection by group A streptococci and *Y. enterocolitica* (Luo et al., 2011, Sun et al., 2009).

Fibrin also provides a scaffold for inflammatory reactions. Binding of the leukocyte integrin receptor $\alpha_M\beta_2$ to the fibrin(ogen) gamma chain residues 390-396 is crucial for the efficient recruitment and activation of neutrophils and macrophages. Mutation of this $\alpha_M\beta_2$ binding region does not affect coagulation, but strongly reduces the clearance of *S. aureus* (Flick et

al., 2004a, Flick et al., 2004b) and increases mortality of group A streptococcal infection (Sun et al., 2009). Also, in a pneumonic plague model, successful phagocytic and T cell mediated defense was dependent on the presence of a fibrin matrix surrounding *Y. pestis* bacteria (Luo et al., 2013).

Fibrinolysis and host defense

The interaction between inflammation and coagulation also extends to the fibrinolytic system. Plasmin is involved in many inflammatory reactions such as complement activation, the bradykinin system and migration of leukocytes (Bugge et al., 1996b, Del Rosso et al., 2008). Plg deficient mice show impaired leukocyte migration and have defective wound healing and tissue repair mechanisms (Schuster et al., 2007).

PAI-1 is upregulated in local as well as systemic inflammatory processes (Chung et al., 2009, Lim et al., 2011, Mandl-Weber et al., 2001, Renckens et al., 2007) and PAI-1 levels correlate with mortality in severe sepsis, both in mouse models (Lim et al., 2011, Raeven et al., 2012, Renckens et al., 2007) and in patients (Green et al., 2002, Lorente et al., 2014, Madoiwa et al., 2006, Pralong et al., 1989, Raaphorst et al., 2001, Skibsted et al., 2013). A polymorphism in the PAI-1 gene affects the likelihood of patients with meningococcal infection to develop sepsis or DIC (Binder et al., 2007, Westendorp et al., 1999); for pneumococcal meningitis an association of the polymorphism with increased mortality and cerebrovascular complications was found (Brouwer et al., 2014). The same PAI-1 polymorphism predicts mortality in sepsis patients (Li et al., 2013). The reduction of PAI-1 activation by means of a

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monoclonal antibody against PAI-1 reduced kidney injury and mortality in a model of disseminated intravascular coagulation (Montes et al., 2000).

Although the role of TAFI in infection is less clear, inhibition or absence of TAFI also reduced organ dysfunction in sepsis caused by *P. aeruginosa* or *E. coli* and in endotoxin shock models (Colucci & Semeraro, 2012, Muto et al., 2009, Renckens et al., 2011).

However, despite the strong correlation between elevated antifibrinolytic activity and mortality, elevated PAI-1 levels have been shown to be protective in murine models of *K. pneumoniae* and *H. influenzae* pneumonia as well as in melioidosis by sustaining neutrophil influx towards the infectious focus, thus minimizing bacterial spreading and pathogen-induced damage (Kager et al., 2011, Lim et al., 2011, Renckens et al., 2007, Wang et al., 2013).

This indicates that despite the risks of an unbalanced coagulation, the inhibition of fibrinolysis can also be favorable to combat certain microorganisms. In this respect, it has been shown that Plg deficiency improves outcome in *S. aureus* sepsis, however, only if very high inocula are used (Guo et al., 2011). Increased Plg activation by administration of t-PA or by absence of its inhibitors increases mortality in *B. pseudomallei* and *Y. enterocolitica* sepsis (Kager et al., 2012, Kager et al., 2013, Luo et al., 2011).

Bacteria and the plasminogen activator system

The Plg system constitutes a potent proteolytic potential that pathogens can usurp to promote their own spreading through tissue barriers. For an increasing number of bacteria, the ability to interact with the Plg system has been strongly linked to their pathogenic potential.

Next to their Plg activating potential, many bacterial pathogens also directly activate the coagulation cascade resulting in fibrin deposition. Bacterial substances such as lipopolysaccharide and lipoteichoic acid stimulate coagulation *via* tissue factor secretion, and inhibit anticoagulation and fibrinolysis (Hoogerwerf et al., 2009, Schlichting et al., 1993). Bacteria can induce contact pathway activation as well (nicely reviewed in Nickel & Renné, 2012). Bacterial surface molecules such as the M protein of *S. pyogenes* and curli from *E. coli* can bind high molecular weight kininogen to the bacterial surface. Furthermore, bacteria can release polyphosphate and thus activate factor XII. Some bacterial proteases such as staphopains from *S. aureus* and SpeB from *S. pyogenes* can directly cleave high molecular weight kininogen to release bradykinin.

The most striking example of bacterial specialization to subvert physiological hemostasis is observed in *S. aureus*. The coagulase activity of *S. aureus* is caused by the production of two bacterial prothrombin activators, staphylocoagulase and von Willebrand factor binding protein. Both proteins conformationally activate prothrombin to yield active staphylothrombin, which is able to generate fibrin and provide *S. aureus* bacteria with shelter from the host defense system (Vanassche et al., 2011). In gram-negative bacteria,

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porins prevent the action of the major thrombin inhibitors antithrombin and α_2 -macroglobulin, thus increasing thrombin activation (DiMicco et al., 2005).

Only a few bacterial species directly activate Plg by the secretion of a bacterial Plg activator. However, a large number of pathogenic bacteria are able to bind Plg to their surface, facilitating the conversion to plasmin and protecting the plasmin activity from subsequent inactivation by α_2 -antiplasmin (**Figure 3**). Finally, bacterial infections also influence the activity of the Plg system through increased expression of Plg activators by host cells (Fuchs et al., 1996, Haile et al., 2006, Kenny et al., 2008, Kitadai et al., 2003, Park et al., 1997).

Bacterial plasminogen receptors

The capacity to engage the host plasmin system at the bacterial surface seems to be almost universal.

Interestingly, bacterial PlgRs can be specialized cell surface proteins, but also multifunctional glycolytic enzymes with a partial surface localization (Chhatwal, 2002, Pancholi & Chhatwal, 2003). Binding of the lysine-binding sites in the Plg kringle domains facilitates the activation of Plg to surface-bound plasmin, and protects the resulting plasmin from inactivation (Bhattacharya et al., 2012, Hall et al., 1991, Lahteenmaki et al., 2001, Sanderson-Smith et al., 2012).

In gram-positive bacteria, the most important PlgRs are the glyceraldehyde-3 phosphate dehydrogenase (GAPDH), also known as Plr or SDH in group A, B and C streptococci (Bergmann et al., 2004, Boël et al., 2005, Kinnby et al., 2008, Lama et al., 2009, Matta et al.,

2010, Pancholi & Fischetti, 1992, Seifert et al., 2003, Winram & Lottenberg, 1996). In addition, α -enolase is implicated in surface Plg binding in staphylococci, streptococci, trichomonas, mycoplasma and *B. anthracis* (Agarwal et al., 2008, Bergmann et al., 2001, Chumchua, et al., 2008, Ehinger et al., 2004, Esgleas et al., 2008, Jones & Holt, 2007, Kinnby et al., 2008, Molkanen et al., 2002, Mundodi et al., 2008, Pancholi & Fischetti, 1998, Whiting et al., 2002, Yavlovich et al., 2007,)), and is also present on the surface of eukaryotic cells (Miles et al., 1991). Furthermore, streptococci can bind Plg through PAM (streptococcal M protein) (Berge & Sjobring, 1993, Fu et al., 2008).

The capacity to bind Plg and acquire pericellular proteolytic activity has also been demonstrated in many gram-negative bacteria (Ullberg et al., 1990). Adhesion structures such as fimbriae and flagella are often involved in this interaction (Lahteenmaki et al., 1993, Sjobring et al., 1994). Fimbriae from pathogenic *E. Coli* act as PlgRs (Kukkonen et al., 1998, Parkkinen & Korhonen, 1989). They also bind t-PA (Sjobring et al., 1994) and mediate adhesion to ECM components, thus colocalizing the proteolytic machinery to the ECM. The presence of α -enolase and GAPDH has also been shown in gram-negative organisms and spirochetes (Egea et al., 2007, Floden et al., 2011, Knaust et al., 2007, Sha et al., 2009, Sijbrandi et al., 2005,). The spirochetes *Borrelia* and *Leptospira* furthermore possess an extensive arsenal of specialized surface PlgRs (Brissette et al., 2009, Fuchs et al., 1994, Hallstrom et al., 2010, Hu et al., 1997, Rossmann et al., 2007, Vieira et al., 2012).

The expression of bacterial Plg activators and PlgRs are not mutually exclusive strategies, but rather seem to complement each other. Oral streptococci express both streptokinase and PlgRs (Kinnby et al., 2008). In a model of impetigo, both streptokinase and the PlgR

PAM were necessary to obtain full pathological changes associated with streptococcal skin infection. This suggests a complementary function of Plg activation through streptokinase and the sequestration of plasmin activity to the bacterial surface (Ringdahl et al., 1998, Svensson et al., 2002). In a similar way, staphylokinase and PlgRs are co-expressed in *S. aureus* (Kuusela & Saksela, 1990, Molkanen et al., 2002, Santala et al., 1999).

Bacterial plasminogen activators

1. Streptokinase

Streptokinase is produced by group A, C and G β -hemolytic streptococci. It consists of a single polypeptide chain with a α , β and γ domain. Polymorphisms allow for distinction between clusters 1 and 2 (2a/2b) of streptokinase alleles (Cook et al., 2012, Zhang et al., 2012b). Streptokinase expression is upregulated by the FasBCAX system, a homologue of the agr quorum sensing system in staphylococci (Sun et al., 2012). Interaction of the streptokinase α and γ domain with Plg induces a conformational change that exposes the catalytic site, leading to Plg activation without hydrolysis of the Arg⁵⁶¹-Val⁵⁶² bond (Wang et al., 1998). This streptokinase-plasmin(ogen) complex can consequentially activate other Plg molecules into plasmin. Furthermore, this conformational change protects the streptokinase-plasmin(ogen) complex from inactivation by α_2 -antiplasmin (Cederholm-Williams et al., 1979). However, Plg-activating properties differ between streptokinase gene clusters. Streptokinase cluster type 2b has a weaker Plg-activating effect and is inhibited by α_2 -antiplasmin; this streptokinase variant was less virulent in a mouse model (Cook et al., 2012). In *S. pyogenes* strains with this streptokinase type 2b, there is a strong coinheritance with the major streptococcal surface PlgR, PAM (Zhang et al., 2012b).

In contrast to u-PA and t-PA, streptokinase has a strong species-specificity, as human pathogenic streptococci do not activate murine Plg and *vice versa* (Sun et al., 2004). Streptokinases of non-human streptococci show remarkable species-specificity for the Plg of the animals they infect (Caballero et al., 1999, Leigh et al., 1998, McCoy et al., 1991). *S. uberis*, a major cause of bovine mastitis, even secretes an unrelated streptococcal Plg activator known as PauA or SUPA, which is specific for bovine and ovine Plg (Rosey et al., 1999, Ward et al., 2004, Zhang et al., 2012a).

Mice are naturally resistant to streptococcal skin infection. This can be explained by the species-specificity of streptokinase for human (and not murine) Plg, as expression or addition of human Plg in a mouse model strongly increased the pathogenic potential (Khil et al., 2003, Li et al., 1999, Ly et al., 2014, Sun et al., 2004).

However, both Plg activation by streptokinase and Plg binding by PAM are species-specific and play an important role in group A streptococcal pathogenesis (Fu et al., 2008, Sanderson-Smith et al., 2008, Sun et al., 2012, Svensson et al., 2002). Invasive isolates of group A streptococci efficiently assemble a trimolecular complex of fibrinogen-Plg-streptokinase through interaction with PAM and M-related proteins (McKay et al., 2004).

2. Staphylokinase

The smaller staphylokinase protein, secreted by the majority of pathogenic *S. aureus* isolates, is structurally unrelated to streptokinase. Its sequence is highly conserved and it is carried on a beta-hemolysin-converting bacteriophage (Coleman et al., 1989, Sumby & Waldor, 2003, Van Wamel et al., 2006). Staphylokinase expression is controlled by quorum-

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sensing signals *via* the *agr* gene regulator (Novick, 2003). The large majority of human clinical isolates are staphylokinase positive and most reports find similar or higher prevalence of staphylokinase-producing strains in clinical infections compared to colonization, especially for skin and soft tissue or bone infections (Humphreys et al., 1989, Luedicke et al., 2010, Monecke et al., 2007, Monecke et al., 2009, Piechowicz et al., 2010, Ruotsalainen et al., 2008, Van Wamel et al., 2006, Wieckowska-Szakiel et al., 2007). Staphylokinase production constitutes an adaptation to the human host, as the gene encoding staphylokinase is lost after transition to animals (Katayama et al., 2013, Resch et al., 2013).

Although staphylokinase, like streptokinase, is not a protease, there are some important mechanistic differences. Staphylokinase has only a weak affinity for Plg, and the staphylokinase-Plg complex is enzymatically inactive. In contrast, staphylokinase had a high affinity for binding to traces of plasmin that are present in plasma (Collen et al., 1993, Silence et al., 1993) and the resulting staphylokinase-plasmin complex is enzymatically active and converts Plg into plasmin. The staphylokinase-plasmin complex is readily inactivated by α_2 -antiplasmin, but binding to fibrin or PlgRs *via* its lysine-binding sites, protects staphylokinase-plasmin from inactivation (Lijnen et al., 1991). This has major implications, as it makes staphylokinase a fibrin-specific Plg activator.

Due to this ability to provide fibrin-specific proteolytic activity without systemic Plg activation, staphylokinase has been evaluated as a fibrin-specific thrombolytic agent, in contrast to the non-fibrin-specific streptokinase (Collen, 1998). The protection of the staphylokinase-plasmin complex from inhibition by α_2 -antiplasmin by surface PlgRs or by fibrin – similar to streptokinase type 2b (Cook et al., 2014) – can confine the associated proteolytic activity to specific niches, close to the bacterial colony or the surrounding fibrin.

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13 virulence remained unresolved.
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16 High-level staphylokinase production significantly reduces sepsis mortality, decreases
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18 spread of skin infection and is incompatible with successful biofilm infection (Kwiecinski et
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20 al., 2010, Kwiecinski et al., 2013, Kwiecinski et al., 2014). Most likely, these observations
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22 can be explained by interference of hyperfibrinolysis with bacterial-induced fibrin
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24 formation, which was shown to be an important virulence factor of *S. aureus* in bacteremia,
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26 abscess formation and catheter infection (Cheng et al., 2010, McAdow et al., 2011,
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28 Vanassche et al., 2011, Vanassche et al., 2013). However, in a mouse model with human Plg
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30 expression through adenoviral gene transfer, staphylokinase-mediated Plg activation in case
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32 of normal-level staphylokinase production, was shown to increase lesion size and bacterial
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34 load (Peetermans et al., 2014).
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49 The *Y. pestis* outer membrane protein Pla is a protease that, although structurally unrelated
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51 to mammalian Plg activators, activates Plg by proteolysis of the same Arg⁵⁶¹-Val⁵⁶² peptide
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53 bond. Pla also activates pro-u-PA (Jarvinen et al., 2013). Furthermore, Pla inactivates the
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55 fibrinolytic inhibitors α_2 -antiplasmin, PAI-1 and TAFI (Haiko et al., 2010, Kukkonen et al.,
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57 2001, Valls Seron et al., 2010). Pla is part of a family of outer membrane proteins (omptins)
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59 present in gram-negative bacteria. Homologues in other species include OmpT from *E. coli*
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3 and pgtE from *S. typhimurium*, which however are only very weak Plg activators (Haiko et
4 al., 2009). Remarkably, these omptins also inactivate tissue factor pathway inhibitor, thus
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6 promoting coagulation (Yun et al., 2009). The combination of a procoagulant function with a
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8 fibrinolytic function seems surprising, but underpins the importance of coagulation
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10 deregulation by bacterial pathogens.
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16 Apart from its Plg-activating function, Pla also adheres to laminin present in basement
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18 membranes and in the ECM, localizing plasmin generation near potential substrates
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20 (Lahteenmaki et al., 1998).
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26 The role of Plg activation as a virulence factor has perhaps been most extensively
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28 documented in *Y. pestis* (Korhonen et al., 2013). The virulence of a Pla-negative mutant was
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30 decreased by a factor 1 000 000 after subcutaneous administration (Sodeinde et al., 1992),
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32 while Pla-negative mutants retained virulence when injected intravenously (Sebbane et al.,
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34 2006, Sodeinde et al., 1992). This strongly suggests the importance of Pla for the
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36 dissemination through skin and soft tissue. While Pla-negative *Y. pestis* can cause systemic
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38 spread when inoculated intranasally, these mutants were less capable of inducing
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40 pulmonary infection (Lathem et al., 2007). Plg deficient mice had a strongly reduced
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42 mortality compared to wild-type mice (100-fold increase in LD₅₀) (Goguen et al., 2000).
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46 Nevertheless, the protective effect of Plg gene deletion was not as profound as that of Pla
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48 inactivation, suggesting that at least some of the pathogenic potential of Pla is independent
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50 from its Plg-activating function.
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Mechanisms of plasmin(ogen)-dependent bacterial virulence (Figure 3)

Table 1 and 2 summarize the experimental evidence for the role of Plg activation in bacterial pathogenicity, derived from *in vitro* data (**Table 1**) and animal models (**Table 2**).

1. Extracellular matrix degradation

An increasing body of *in vitro* and *in vivo* evidence shows that bacteria rely on Plg activation to breach through cellular monolayers, degrade ECM and facilitate bacterial spreading.

The involvement of Plg in matrix and basement membrane degradation has been shown for *H. influenzae* and *S. pneumoniae*. In addition, *N. meningitidis* is also able to bind human Plg (Knaust et al., 2007, Ullberg et al., 1992). These three pathogens are the most important causes of bacterial meningitis. *In vivo* experiments have shown that Plg activation by *Borrelia spp.* facilitates penetration of the blood brain barrier (Gebbia et al., 1999, Nordstrand et al., 2001). The opportunistic pathogen *C. neoformans*, a yeast that typically causes meningitis in AIDS patients, also uses Plg to breach through matrix barriers (Stie et al., 2009). The capacity to engage plasmin activity may be crucial for the penetration of bacteria into the central nervous system, highlighting the clinical relevance of this pathogen-host interaction.

In a staphylococcal skin infection model, activation of Plg by staphylokinase and secondary MMP-activation led to increased subcutaneous spreading of infection (Peetermans et al., 2014). In group A streptococcal infection, necrotic skin lesions and increased mortality were observed if both streptokinase and human Plg were present (Khil et al., 2003, Sanderson-Smith et al., 2008).

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2. Escape from innate immunity

Host plasmin-mediated proteolysis can also be beneficial for bacteria by degrading peptides and proteins involved in first-line defense mechanisms. These include complement components and antimicrobial peptides such as defensins.

Surface-bound plasmin activity in *Borrelia spp.* has an anti-opsonic effect by decreasing C3b deposition (Koenigs et al., 2013). In *F. tularensis*, removal of bound IgG by surface-associated plasmin leads to decreased phagocytosis by macrophages (Crane et al., 2009). A similar protective effect was seen in group A streptococci and *S. aureus* (Koch et al., 2012, Ly et al., 2014, Rooijakkers et al., 2005, Siemens et al., 2011). Staphylokinase induces a rapid release of α -defensins but also subsequently degrades and thus neutralizes their antimicrobial effect (Jin et al., 2004). Cathelicidin, another antimicrobial peptide, is upregulated in the airways after bacterial infection. Staphylokinase binds cathelicidin and this interaction enhances staphylokinase-mediated fibrinolysis (Braff et al., 2007).

3. Fibrin degradation

As noted above, activation of coagulation and the resulting encapsulation of bacteria with fibrin can be considered as part of the innate immune response to infection. Bacterial-induced plasmin activity can degrade this fibrin network to promote bacterial spreading.

Plg deficiency dramatically reduced mortality from *Y. pestis* in mice, but this survival benefit was lost in mice with a combined fibrinogen and Plg deficiency that failed to generate inflammatory infiltrates (Degen et al., 2007).

S. aureus biofilms on foreign surfaces contain large amounts of fibrin, generated by host thrombin as well as bacterial staphylothrombin activity. As the bacterial population

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3 increases, quorum-sensing mechanisms are activated, and staphylokinase production will
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5 increase. This will shift the balance from staphylocoagulase activity resulting in fibrin
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7 formation, to local degradation of fibrin mediated by the fibrin-specific plasminogen
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9 activator of *S. aureus*. Plg activation by staphylokinase dissolves staphylococcal biofilm
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11 matrix and thus allows detachment of bacteria to disseminate to new sites (Kwiecinski et al.,
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13 2014, Otto, 2004).
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24 Interfering with bacterial plasminogen activation: therapeutic potential?

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26 Despite promising data from studies in knock-out mice or with mutant bacteria (Table 1-2),
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28 few therapeutic applications have made the transition to patient care, with the exception of
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30 the use of streptokinase and staphylokinase as thrombolytic drugs in cardiovascular
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32 thrombosis (Collen, 1998). There are several explanations for this lack of translation to the
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34 clinical practice of infectious diseases.
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38 First, many bacteria have redundant mechanisms to interact with Plg, e.g. combining several
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40 types of surface PlgRs with the secretion of a bacterial plasminogen activator. Also, some
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42 virulence factors have pleiotropic functions beyond their Plg activating potential. For
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44 example, streptococcal M protein does not only interact with Plg, but also with fibrinogen,
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46 kininogen, immunoglobulins, C4b binding protein, factor H and factor H-like protein, thus
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48 accounting for its role as an antiphagocytic factor (Smeesters et al., 2010). M protein
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50 vaccines are currently under development, however their protective efficacy relies on the
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52 production of opsonizing antibodies rather than on the interference with Plg binding to *S.*
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pyogenes (Dale et al., 2013). The same holds true for borrelial vaccines directed to OspC

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(Scheiblhofer et al., 2003, Wagemakers et al., 2014) and pneumococcal vaccines based on α -enolase (Adrian et al., 2004).

Secondly, some major virulence factors such as Pla from *Y. pestis* have failed as vaccine antigens. The protective efficacy of Pla-based immunization in animal models was dependent on the type of infection (bubonic vs. pneumonic plague) and the rodent model used (Erova et al., 2013, van Lier et al., 2014).

Thirdly, inhibition of fibrinolytic activity might increase fibrin encapsulation of bacteria, resulting in chronic and persistent infections. Remarkably, some bacteria interact with both coagulation and fibrinolysis. This apparent contradiction can be understood by the regulated expression of different virulence factors, depending on the growth phase of the bacteria. In some stages or types of infection, preventing coagulation cascade activation rather than inhibiting fibrinolysis, could be a more effective therapeutic strategy. Examples are interference with bacterial activation of the contact pathway (Oehmcke et al., 2009) or bacterial prothrombin activation (Peetermans et al., 2015). Importantly, catheter lock therapy with rt-PA was shown to prevent catheter related bacteremia in hemodialysis patients, probably by reducing fibrin deposition and bacterial adhesion (Hemmelgam et al., 2011). Some case reports and small trials describe the use of thrombolytic agents in the adjunctive treatment of fibrinopurulent infections of the pericardium, catheters or other foreign bodies (Bigam et al., 2008, Innes et al., 1994, Levitas et al., 2015, Masuya et al., 2006, Reznikoff et al., 2003, Wiggins et al., 2007). In fact, exogenously administered fibrinolytics are used here to break down the fibrin-encapsulated bacterial colony adhering to a foreign body; a strategy that is utilized on a smaller scale by *S. aureus* bacteria that produce staphylokinase to escape from an established biofilm when quorum sensing mechanisms are activated.

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4 However, in acute locally invasive or tissue destructive infections such as group A or B
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6 streptococcal infections, where proteolytic activation has been shown to be a major
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8 virulence factor, it is tempting to speculate for an adjunctive effect of plasmin inhibitors in
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10 their treatment. In a study by Khil and colleagues (Khil et al., 2003), coadministration of
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12 group A streptococci with human Plg led to necrotic skin lesions in mice. Those lesions were
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14 almost completely absent if an irreversible plasmin inhibitor was added to the inoculum. In
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16 a mouse model for group B streptococcal neonatal meningitis and sepsis, administration of
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18 epsilon-aminocaproic acid to the drinking water of the mothers was associated with less
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20 mortality, less tissue invasion of brain and lungs and lower organ bacterial load in neonates
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22 (Magalhaes et al., 2013). Clinical data on the potential use of plasmin inhibitors in rapidly
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24 spreading skin and soft tissue infections are not yet available.
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31 A few recent publications have explored new pathways for therapeutic targeting of bacterial
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33 activation of host Plg. For examples, a promising strategy used a small molecule that
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35 interferes with bacterial gene regulation of *S. pyogenes* (possibly through inhibition of
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37 FasBCAX) and reduces streptokinase expression almost fourfold (Sun et al., 2012). Targeting
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39 the regulation of virulence factor expression is being investigated for *Y. pestis* and *S. aureus*
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41 infections as well (Gray et al., 2013, Murray et al., 2014, Zhan et al., 2008).
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Conclusion

While the coagulation system provides a first barrier against physical and biological damage, overwhelming coagulation activation increases mortality in severe inflammatory states. The fibrinolytic system protects against unwanted fibrin formation, but is also crucial for many other physiological and pathological processes that require cell migration. The balance between adequate coagulation and fibrinolysis is tightly connected to inflammation and the immune system.

The virulence of bacteria capable of causing human disease in part depends on the evolved ways to bypass innate immunity. The available evidence shows that in many cases tissue damage, bacterial spreading and even lethal potential does not result directly from bacterial enzymes, but from the host's own built-in proteolytic system that is subverted by the pathogen. Plasmin-mediated fibrinolysis helps to overcome the initial fibrin deposition that acts as first-line host response mechanism. Subsequent spreading through tissue barriers, as well as degradation of peptides of the innate immune system can be mediated by Plg activation, which is facilitated or induced by the pathogen's PlgRs and Plg activators. The understanding of these mechanisms may lead to the development of novel therapeutic strategies.

Abbreviations

ECM = extracellular matrix

Plg = plasminogen

PlgR(s) = plasminogen receptor(s)

t-PA = tissue-type plasminogen activator

u-PA = urokinase

u-PAR = u-PA receptor

PAI = plasminogen activator inhibitor

TAFI = thrombin-activatable fibrinolysis inhibitor

GAPDH = glyceraldehyde-3 phosphate dehydrogenase

PAM = streptococcal M protein

MMP = matrix metalloproteinase

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Table 1. *In vitro* evidence for the role of plasminogen activation in bacterial virulence.
Plg = plasminogen; PA = plasminogen activator.

Pathogen	ECM degradation and cellular permeation (actor)	Immune evasion (actor)
<i>Yersinia pestis</i>	Degradation of laminin, heparan sulphate proteoglycans and lung ECM (Pla expressed in <i>E. coli</i> + human Plg) (Lahteenmaki et al., 1998)	
<i>Staphylococcus aureus</i>	Penetration of Matrigel, keratinocyte layer and skin biopsy specimen (staphylokinase + human Plg) (Kwiecinski et al., 2013)	Removal of opsonizing IgG and C3b from bacterial surface; protection from Fc-receptor mediated neutrophil phagocytosis (staphylokinase + human Plg) (Rooijackers et al., 2005)
	Degradation of synthetic collagenase substrate, via pro-MMP-1 activation (surface bound human Plg + PA) (Santala et al., 1999)	C3/C3b degradation; protection from complement mediated killing (surface bound Plg, to Sbi and Efb surface proteins, + staphylokinase or host PA) (Koch et al., 2012)
<i>Group A streptococci</i>	Pericellular penetration of pharyngeal cells (surface bound Plg + PA) (Pancholi et al., 2003)	C3b degradation; protection from complement mediated killing by polymorphonuclear leucocytes (surface bound human Plg +/- SK) (Ly et al., 2014)
		Protection from macrophage killing (surface bound human Plg/plasmin) (Siemens et al., 2011)
<i>Group B streptococci</i>	Degradation of fibronectin (surface bound Plg + PA; bacteria grown in human plasma) (Magalhaes et al., 2007)	
	Adhesion to and invasion of human brain microvascular endothelial cells (surface bound human Plg + PA; bacteria grown in plasma) (Magalhaes et al., 2013)	
<i>Group C streptococci</i> (<i>S. dysgalactiae</i> <i>ssp. equisimilis</i>)	Degradation of fibronectin (surface bound human Plg + PA) (Bergmann et al., 2011)	

<i>Streptococcus pneumoniae</i>	Degradation of Matrigel (surface bound Plg + PA) (Eberhard et al., 1999)	C3/C3b cleavage (surface protein Tuf or PepO + human Plg + PA) (Mohan et al., 2014, Agarwal et al., 2013)
	Migration through lung epithelial cell or endothelial cell layer by disruption of intercellular junctions and VE-cadherin degradation (surface bound human Plg + PA) (Attali et al., 2008a)	
	Deletion of plasminogen binding motif in α -enolase reduces degradation of laminin, lung ECM and Matrigel (surface bound human Plg + PA) (Bergmann et al., 2005)	
	Degradation of fibronectin and laminin (surface protein PepO + human Plg + PA) (Agarwal et al., 2013)	
	Deletion of plasminogen binding protein CBPE reduces crossing of Matrigel (surface bound human Plg + PA) (Attali et al., 2008b)	
<i>Streptococcus mutans</i>	Degradation of fibronectin (surface bound human Plg +/- PA; or bacteria grown in plasma or serum) (Jones and Holt, 2004)	
<i>Borrelia burgdorferi</i>	Degradation of fibronectin, laminin, vitronectin and ECM (surface bound human Plg + PA) (Coleman et al., 1999, Coleman and Benach, 2000)	Degradation of C3b and C5 and reduction of complement-mediated bacteriolysis (surface protein BBA70 + Plg + PA) (Koenigs et al., 2013)
	Permeation of endothelial cell monolayer (surface bound human Plg + PA) (Coleman et al., 1995)	
	Permeation of brain microvascular endothelial cells (surface bound human Plg + PA produced by endothelial cells) (Grab et al., 2005)	

<i>Borrelia afzelii</i> , <i>Borrelia spielmanii</i>		Degradation of C3b (surface protein CspA + Plg + PA) (Hammerschmidt et al., 2014)
<i>Borrelia recurrentis</i>		Decrease in C3b deposition and protection from complement-mediated bacteriolysis (surface protein hcpA + Plg + PA) (Grosskinsky et al., 2009)
<i>Leptospira interrogans</i>	Degradation of fibronectin (surface-bound human Plg + PA) (Vieira et al., 2009)	Removal of opsonizing C3b and IgG, C3b degradation, protection from complement mediated killing (surface bound Plg + PA) (Vieira et al., 2011)
	Permeation of endothelial cells (surface bound human Plg +/- PA) (Vieira et al., 2013)	
<i>Bacillus anthracis</i>	Degradation of fibronectin (surface bound human Plg +/- PA) (Agarwal et al., 2008)	Cleavage and degradation of C3b (surface bound human Plg + PA) (Chung et al., 2011)
<i>Haemophilus influenza</i>	Degradation of fibronectin, laminin, endothelial cell ECM and migration through Matrigel (surface bound human Plg + PA) (Virkola et al., 1996)	Degradation of C3b (surface protein PE + human Plg + PA) (Barthel et al., 2012)
<i>Salmonella enterica</i> var. <i>typhimurium</i>	Degradation of laminin, endothelial cell basement membrane and penetration through Matrigel (surface bound human Plg + PA) (Lahteenmaki et al., 1995)	Increased C3b cleavage (surface protein PgtE +/- human Plg / plasmin) (Ramu et al., 2007)
<i>Francisella tularensis</i>	Degradation of fibronectin (surface-bound human Plg + PA) (Clinton et al., 2010)	Removal and degradation of opsonizing IgG, leading to decreased uptake by macrophages and less TNF- α and IL-6 production (surface bound Plg + PA; or plasmin) (Crane et al., 2009)
<i>Mycoplasma fermentans</i>	Invasion of HeLa cells (surface bound Plg + PA) (Yavlovich et al., 2004)	

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Table 2. *In vivo* evidence for the role of plasminogen activation in bacterial virulence.

WT = wild type; SC = subcutaneous; IV = intravenous; Plg = plasminogen; Fib = fibrinogen; IN = intranasal; SAK = staphylokinase, huPlg = human plasminogen; SK = streptokinase; ID = intradermal; u-PA = urokinase; PAM = streptococcal M protein; IP = intraperitoneal; IT = intratracheal.

Pathogen	Bacteria	Host (mice)	Model / parameter studied	Effect	Reference
<i>Yersinia pestis</i>	WT vs. Pla-		SC and IV / mortality, metastatic infection	Pla- <i>Y. pestis</i> less virulent (10^6 -fold increase LD ₅₀) in SC, but not in IV model	(Sodeinde et al., 1992)
	WT vs. Pla-		Flea-borne infection - bubonic vs. systemic plague / mortality	Pla- <i>Y. Pestis</i> can cause fatal systemic plague but not bubonic plague	(Sebbane et al., 2006)
	WT vs. Pla-	WT vs. Plg -/- vs. Fib -/- vs. Fib -/-Plg -/-	SC / mortality	Plg-/- mice are less susceptible to infection with <i>Y. Pestis</i> (100-fold increase LD ₅₀), Fib -/- mice are more susceptible to infection Reduced virulence of <i>Y. pestis</i> in Plg-/- mice is partially reversed by fibrin depletion	(Goguen et al., 2000, Degen et al., 2007)
	WT vs. Pla-		IN - pneumonic plague	Pla- <i>Y. Pestis</i> show reduced proliferation in lungs, reduced fibrin encapsulation and inflammatory damage, and increased survival	(Lathem et al., 2007)
<i>Staphylococcus aureus</i>	SAK- vs. SAK+ vs. SAK overproducing	WT vs. huPlg+	IV / mortality	SAK producing strains are less virulent, if human Plg present	(Kwiecinski et al., 2010)
	SAK- vs. SAK+ vs. SAK overproducing	WT vs. huPlg+ neutropenic	SC / skin infection	SAK overproducing strain causes smaller and more open lesions	(Kwiecinski et al., 2013)
		WT vs. huPlg expression	SC / skin infection	Presence of human Plg increases lesion size and bacterial load	(Peetermans et al., 2014)

Group A <i>streptococci</i>	SK+ vs. SK-	WT vs. huPlg + Fib depletion (Ancrod)	SC or IV / mortality, metastatic infection	Presence of human Plg increases mortality and systemic infection in SC, but not in IV model. Increased mortality in Fib-depleted mice. SK- mutant is less virulent in huPlg + mice (difference not significant in WT mice)	(Sun et al., 2004)
	SK+ vs. SK-	Treatment with human Plg	SC / skin lesions and mortality	Presence of human Plg enhances virulence, counteracted by plasmin inhibitor. Effect more pronounced in SK+ vs. SK- GAS	(Khil et al., 2003)
	Preincubation with human plasma or huPlg, Fib and SK	WT vs. Plg -	ID skin air sac / skin lesions, mortality	Preincubation with human Plg, Fib, SK or human plasma + SK increases virulence in Plg+, but not in Plg- mice, pointing to the role of surface bound plasmin and secondary activation of murine Plg	(Li et al., 1999)
	SK+ vs. SK-	WT vs. huPlg + vs. u-PA -	ID / mortality, metastatic infection, bacteremia	Presence of human Plg and u-PA increases mortality and dissemination. Increased virulence of SK+ strain in uPA-/- background	(Sanderson- Smith et al., 2013)
	SK+ vs. SK- PAM+ vs. PAM-	human foreskin graft in SCID mice	Infection of damaged skin / skin lesions	Both PAM-deletion and SK-deletion reduce pathological changes. Effect of SK-deletion more pronounced	(Svensson et al., 2002)
	WT vs. PAM -	WT vs. huPlg +	SC / mortality, metastatic infection	PAM- mutant is less virulent in huPlg + mice (difference not significant in WT mice)	(Sun et al., 2004)
	WT vs. prp -	huPlg +	SC / mortality	Inactivation of Plg-binding protein prp reduces virulence in huPlg + mice	(Sanderson- Smith et al., 2008)
	WT vs. prp-	huPlg +	ID / lesion bacterial load	Increased bacterial survival, less C3b deposition and neutrophil accumulation	(Ly et al., 2014)
	WT vs. speB-	huPlg +	SC / mortality, bacteremia	SpeB loss results in increased plasmin acquisition and increases mortality and invasiveness in huPlg + mice	(Cole et al., 2006)

Group B streptococci (<i>S. agalactiae</i>)	incubation with human Plg and t-PA		IP / liver count of bacteria	Pre-incubation with human Plg and t-PA enhances virulence and decreases clearance from liver	(Magalhaes et al., 2007)
		EACA treatment of mothers	IP - neonatal mouse meningitis / organ bacterial load	More penetration of blood brain barrier, reduced by EACA treatment	(Magalhaes et al., 2013)
<i>Streptococcus pneumoniae</i>	WT vs. α -enolase mutant		IN - pneumonia model / mortality	Mutation in Plg-binding motif of α -enolase reduces virulence	(Bergmann et al., 2003)
<i>Borrelia burgdorferi</i>		WT vs. Plg -/-	Tick-borne infection – SC / dissemination in tick and in mice	Plg-deficiency decreases dissemination in ticks, leads to longer attachment of nymphs and reduces spirochetemia in mice	(Coleman et al., 1997)
	WT vs. OspC mutant		Tick-borne infection / organ bacterial load	Low-affinity binding of Plg in OspC mutant reduces invasiveness (heart, joint, ear skin)	(Lagal et al., 2006)
		Active immunisation to enolase	SC and tick-borne infection / acquisition in tick	Lower acquisition of <i>Borrelia</i> in tick	(Nogueira et al., 2012)
<i>Borrelia</i> from patient with relapsing fever		WT vs. Plg -/-	SC / penetration in brain and heart	Plg deficiency reduces invasion of brain and heart	(Gebbia et al., 1999)
<i>Borrelia crocidurae</i>		WT vs. Plg -/-	IP / bacteremia pathological changes	Plg -/- mice have equal levels of circulating bacteria but reduced invasion and inflammation of brain and kidney	(Nordstrand et al., 2001)
<i>Aeromonas hydrophila</i>		Immunization to enolase	IV / mortality, bacterial load	Immunized mice are protected from lethal challenge and have reduced bacterial loads in liver and spleen	(Sha et al., 2009)
<i>Mycobacterium avium</i>		WT vs. Plg -/-	IT / organ bacterial load	Absence of Plg leads to earlier dissemination (higher liver and spleen CFU counts after one week) and more fibrin deposition and neutrophil infiltration	(Sato et al., 2003)

Figure 1. Overview of the coagulation and fibrinolytic system with special reference to plasmin substrates and fibrinolytic inhibitors.

PlgRs = plasminogen receptors; t-PA = tissue type plasminogen activator; u-PA = urokinase; u-PAR = u-PA receptor; PAI-1 = plasminogen activator inhibitor 1; α2AP = alpha-2-antiplasmin; α2M = alpha-2-macroglobulin; MMPs = matrix metalloproteinases; ECM = extracellular matrix.
258x172mm (300 x 300 DPI)

Figure 2. Domain structure of plasminogen.

Plasminogen (Plg) contains five kringle domains and an active serine protease site. Disulfide bridges are marked in grey. Plg is activated by plasminogen activators (Plg activators) through cleavage between Arg⁵⁶¹ and Val⁵⁶², as indicated. This leads to formation of plasmin, consisting of a heavy chain and a light chain. Conversion of Glu-Plg to Lys-Plg involves removal of the C-terminal activation peptide (AP) by plasmin.

191x129mm (300 x 300 DPI)

Figure 3. Mechanisms of plasminogen activation by bacteria and its role in bacterial virulence.

A. Bacteria can bind plasminogen (Plg) to their surface, using specialized surface plasminogen receptors (PlgRs) that interact with the kringle domains of Plg. Surface-bound Plg can be easily activated by plasminogen activators (t-PA or u-PA); the resulting surface-associated plasmin activity is protected from alpha-2-antiplasmin (α 2AP) inhibition. Alternatively, some bacteria produce a specific bacterial plasminogen activator (Plg activator), such as Pla protease (from *Y. pestis*) and the nonproteolytic Plg activators streptokinase (SK from group A, C and G streptococci) and staphylokinase (SAK from *S. aureus*).

B. The plasmin activity generated by bacteria is involved in bacterial invasion through degradation of extracellular matrix (ECM); in escape from the immune system (immune evasion), e.g. through removal of opsonizing complement factors and immunoglobulins; and in degradation of the fibrin that is formed around the bacterial colony.

297x234mm (300 x 300 DPI)

Figure captions

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