Immune Evasion by Pseudomonal Proteases

Bart Willem Bardoel

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Immune Evasion by Pseudomonal Proteases

Ontwijking van het immuun systeem door
proteases van *Pseudomonas*

(met een samenvatting in het Nederlands)

Proefschrift

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Bart Willem Bardoel

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Promotor: Prof.dr. J.A.G. van Strijp

Co-promotor: Dr. K.P.M. van Kessel

Voor mijn ouders

Commissie:	Prof.dr. D. de Kleyn
	Prof.dr. L. Meyaard
	Prof.dr. C.M.J. Pieterse
	Prof.dr. J.P.M. van Putten
	Prof.dr. J.P.M. Tommassen
Paranimfen:	Alex Laarman
	Bas Surewaard

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General Introduction

The molecular battle between host and bacterium:

Recognition in innate immunity

Bart Bardoel and Jos van Strijp

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Innate immunity

In contrast to the impression textbooks often give, it is important to note that our innate immune system is not solely designed to eliminate pathogens. The primary task of innate immunity is fast elimination of all invading microorganisms; the ones that escape this removal can become pathogens. Therefore innate recognition involves sensing of many millions of species of bacteria, fungi, parasites and viruses. Since innate immunity is embedded in our genome and with less than 30,000 genes this seems an impossible task. However the answer is simple: In order to recognize all microbes our innate immune system senses evolutionary conserved structures; the molecular signatures that are present in microbes and not in our own body. Our innate immune system contains three important effector mechanisms that allow rapid and efficient removal of bacteria; professional phagocytes, the complement system and antimicrobial peptides.

Antimicrobial peptides

Antimicrobial peptides and antimicrobial proteins directly recognize microbial structures and lyse the bacterial membrane, thereby killing the bacterium in seconds to minutes. However to be effective the concentrations of these peptides have to reach relatively high concentrations. Local high concentrations are found in the gut and inside phagocytes (granules) [1,2,3].

Complement system

The complement system consists of roughly 30 proteins in our blood. This system is present in all vertebrates and the higher invertebrates, and the individual components are highly conserved between species. Activation triggers a proteolytic cascade of complement proteins, resulting in downstream effector functions such as phagocytosis and killing of Gram-negative bacteria. Three different routes initiate complement activation, the classical pathway (CP) (Fig. 1), the lectin pathway (LP) and the alternative pathway (AP). In the CP C1q recognizes antigen antibody complexes. C1q recruits C1r, a serine protease, that cleaves C1s, the third component of the C1 complex. C1s has proteolytic activity for the complement proteins C4 and C2 resulting in an instable complex called C3 convertase that can cleave the central complement protein C3. Initiation of the lectin pathway occurs by binding of mannose binding lectin (MBL) or ficolins to carbohydrate structures of microorganisms. Upon binding of these carbohydrate binding molecules MBL-associated serine proteases are recruited and activated, which cleave C4 and C2, resulting in formation of a C3 convertase. The alternative pathway is spontaneously activated by hydrolysis of C3 and also serves as an amplification loop for C3 cleavage.

Cleavage of C3 splits the molecule in C3a, a potent inflammatory mediator that triggers the C3a receptor on cells [4], and C3b that is attached to a surface via a highly reactive thioester. C3b acts as an opsonin that labels bacteria for phagocytosis by neutrophils. C3b can also bind to C3 convertases, resulting in the formation of a C5 convertase. This protein complex cleaves C5 in C5a, a potent chemoattractant that triggers the C5a receptor [4], and C5b which binds subsequently C6, C7, C8 and multiple C9 molecules to form the membrane attack complex. C9 forms a ring structure that is inserted in the membrane of Gram-negative bacteria resulting in pore formation and lysis.

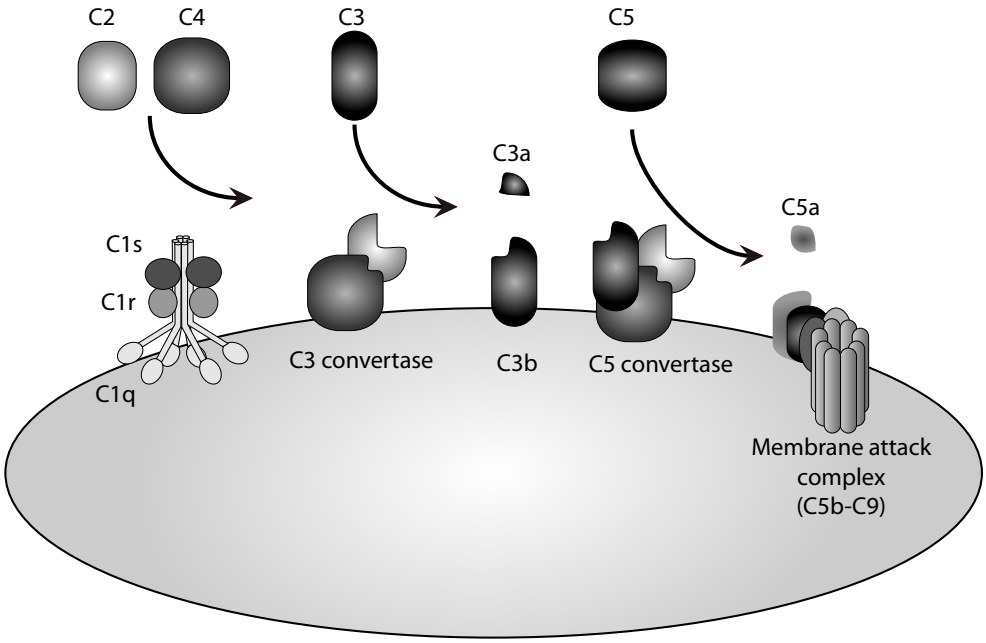

Figure 1. Overview of the complement system
Simplified overview of the classical pathway of the complement system and the deposition of complement proteins on the bacterial surface.

Phagocytes

Phagocytes (such as neutrophils, monocytes and macrophages) can ingest and kill bacteria within a minute. Of these cells, neutrophils are most abundant (50% of all white blood cells in humans) and best equipped to fight bacteria. Killing is achieved by a vast array of proteases, lipases, antimicrobial peptides, amidases, or by a complete arsenal of highly reactive oxygen radicals, but often by a combination of all of these factors working synergistically. At the site of infection, phagocytes immediately recognize hostile bacteria, either directly by surface receptors that are able to recognize structurally conserved molecular patterns on many bacterial surfaces, or by opsonin receptors to recognize a coating of complement proteins or other plasma proteins on the bacterial surface. Triggering of these receptors initiates phagocytosis and killing. Bacteria can also be opsonized by antibodies. These are in turn recognized by the Fc receptor, at the constant fragment (Fc) of the immunoglobulin. After recognition and binding to the Fc receptor, phagocytes are activated, the bacteria are engulfed and a phagosome is formed. Uptake of the bacteria is not only stimulated by the receptors that directly recognize the bacteria, but also by cellular activators such as C5a. The phagosome acidifies, because toxic molecules are pumped into the phagosome. The phagosome fuses with the lysosomes, exposing the engulfed bacteria to proteolytic enzymes. Next to an array of proteases and cell wall degrading enzymes, reactive oxygen species and nitrogen intermediates damage and eventually kill the bacteria.

Innate recognition of evolutionary conserved bacterial structures

A schematic representation of the Gram-negative and Gram-positive cell wall structure is presented in Figure 2. Gram-negative bacteria have two lipid bilayer membranes and a periplasmic space in between. Within that space a peptidoglycan layer is present supporting structure and rigidity. In the outer leaflet of the outer membrane endotoxin or lipopolysaccharide (LPS) is the main constituent. In Gram-positive bacteria the peptidoglycan layer is considerably thicker than in Gram-negatives, while the outer membrane is absent. Figure 3 shows a simplified overview of conserved bacterial structures that are distinct from eukaryotic structures, and are therefore the perfect targets for self from non-self recognition by eukaryotic innate immune receptors. Specific cell wall components as peptidoglycan and teichoic acids are obvious targets but also flagellin, LPS and lipoproteins serve as targets for the innate immune system.

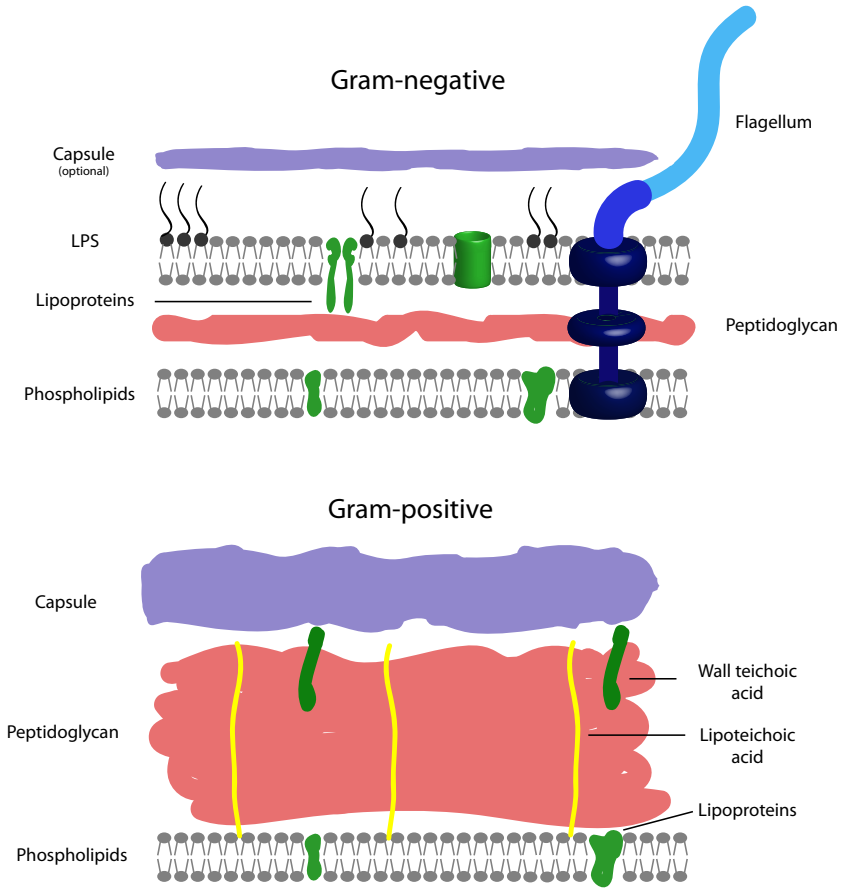

Figure 2. Overview of the composition of Gram-negative and -positive cell walls
The cell wall of Gram-positive bacteria consists of one membrane layer and a thick peptidoglycan layer. Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane, which contains lipopolysaccharide (LPS). Some Gram-negative and –positive bacteria have a flagellum and/or a capsule.

These last three conserved microbial signatures are recognized by Toll-like receptors (TLRs), a prototype family of pathogens sensors. These receptors consist of an extracellular leucine-rich repeat domain involved in ligand recognition, a transmembrane region, and a Toll/Interleukin 1 receptor domain. TLRs have been extensively studied in the last decade, and structural studies between ligand and receptor have provided insight in the molecular recognition of various conserved microbial molecules. For activation of TLRs, dimerization is required, which triggers recruitment of intracellular adapter proteins and initiation of a signaling cascade. This results in production of cytokines, activation and recruitment of various inflammatory cells to the site of infection. Next to Toll-like receptors, formylpeptide receptors and a wide variety of lectins and lectin-like molecules assure direct recognition. Indirect recognition occurs via activation of the complement system and the respective recognition of the opsonized bacterium by different complement receptors. Below we describe the most important bacterial evolutionary conserved structures, their respective counterparts in innate recognition, and the molecular mechanisms of recognition.

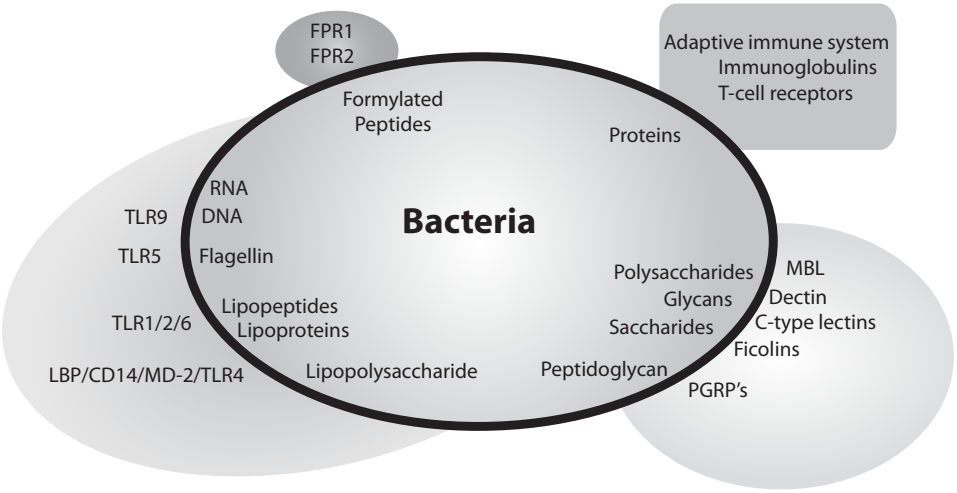

Figure 3. Innate recognition of conserved bacterial structures
Simplified overview of bacterial conserved structures that are recognized by the immune system. The innate immune system recognizes a wide variety of molecules from the protein flagellin to various carbohydrates.

Lipopolysaccharide (LPS)

LPS is a major component of the outer membrane of Gram-negative bacteria. LPS is a complex glycolipid that consists of a hydrophobic lipid A domain, an oligosaccharide core and a distal polysaccharide (O-antigen) (Fig. 4). The lipid A domain generates a strong innate immune response [5], and (in high amounts) is also responsible for endotoxic shock and sepsis [6]. Recognition of LPS by the innate immune system occurs via LPS-binding protein (LBP), which solubilizes and then transfers the monomeric form of LPS to CD14. CD14 (a phospholipid transfer molecule) presents LPS to the TLR4-MD2 receptor complex to trigger dimerization, resulting in activation of downstream signaling.
The strong immunogenic lipid A part is the minimal structure of LPS that is required for the integrity of the outer membrane and is not surface-exposed. The O-antigen is hydrophilic

and the most surface-exposed part of LPS. The O-antigen differs between bacteria in sugar linkage, repetitive numbers, in contrast to lipid A, which structure and synthetic pathway are highly conserved in Gram-negative bacteria. The lipid A domain is characterized by four to seven acyl side chains with slightly different lengths and phosphorylation of one or two glucosamines. LPS from *E. coli* growing under normal laboratory conditions contains a hexa-acylated lipid A with a 2-keto-3-deoxyoctonoic acid (KDO) modification (an unusual sugar specifically found in the core region of LPS), which is necessary for integrity of the outer membrane [5]. The lipid A structure of *E. coli* consisting of these six acyl chains and two phosphate groups is one of the most potent stimulators of TLR4. The variety in lipid A structures between bacteria explains the difference in biological activity of LPS. By changing the number of acyl groups from six to five or seven, the biological activity of LPS is decreased with about 100-fold. Moreover, lipid A with only four acyl groups [7] (which is an intermediate in LPS biosynthesis) is an antagonist of human TLR4. Harsh conditions such as high temperatures probably favors the linkage of six acyl side chains to lipid A [5], because this enhances the stability of the outer membrane.

Recent structural data confirmed that LPS interact with the hydrophobic pocket of MD-2 with five of its six lipid chains, while the remaining chain interacts with the surface of MD-2 [8]. Lipid IVa consists of a different glucosamine backbone, which is rotated by 180 degrees in comparison with LPS that contains six lipid chains, and thereby interchanging the phosphate groups. This different orientation results in the antagonistic effect of lipid IVa. TLR4 contains a domain that is hypervariable across species, in contrast to the other domains of TLR4, which are conserved [7]. This hypervariable domain is responsible for the difference in reactivity between mouse and human TLR4 to penta- or hexa-acylated LPS of *P. aeruginosa*.

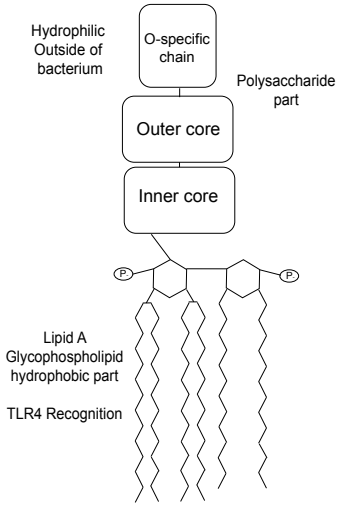

Figure 4. Schematic representation of lipopolysaccharide
LPS consists of, from the outside to the inside of the bacterium, the hydrophilic O-antigen, a polysachharide and a lipid A domain. The biological activity resides within the hydrophobic lipid A that anchors LPS in the bacterial outer membrane. Lipid A differs in acyl side chain number and length, however the overall structure and synthetic pathway is conserved among Gram-negative bacteria.

Lipopeptides

Bacteria use lipoproteins to anchor hydrophilic molecules to their membrane, where they have important functions in bacterial pathogenesis [9]. Gram-positive bacteria and mycoplasma produce lipoproteins that contain diacylated lipopeptides. Gram-negative bacteria and mycobacteria have triacylated lipoproteins [10]. Lipoproteins all share a lipid modification of the N-terminal cysteine residue, which connects the protein to the hydrophobic bacterial membrane. This lipid modification is unique and is present in over 2000 bacterial proteins [11]. Except for this conserved lipid modification in the N-terminus, lipoproteins have no similarities in sequence or function.

TLR2 forms heterodimers together with TLR6 or TLR1 and can recognize different lipoproteins with these receptor complexes. The N-terminal cysteine is essential for TLR2 activity, synthetic peptides that lack almost all residues except this group maintain full activity. TLR1/2 recognizes both diacylated and triacylated lipid modifications of the N-terminal cysteine residue, while triacylated can only activate TLR2/6 [12]. TLR2 can also form homodimers and recognize lipoproteins independent of TLR1 and TLR6 [13], such as the synthetic peptide Pam2Cys. Recognition of lipoproteins by TLR2 heterodimers not only depends on the number of acyl chains, but also the amide-bound acyl residue, the two ester-bound acyl residues and the sequence of the peptide within the lipoprotein [13]. Despite the fact that heterodimerization expands the spectrum of recognized ligands, the downstream signaling and production of cytokines does not change. Recent crystal structures of TLR2 in complex with TLR6 or TLR1 together with different ligands show insight in the recognition of lipoproteins at the molecular level. Interaction between the lipid chains of lipoproteins and the hydrophobic pocket of TLR2 is the driving force for activation. Triacylated lipoproteins have three acyl groups at the N-terminal cysteine, two of them interact with the hydrophobic pocket of TLR2 and one with TLR1. These three acyl chains form a bridge between TLR1 and TLR2 resulting in heterodimerization.

In the last decade, a wide variety of TLR2 ligands have been described with different biological activities. The most powerful activators of TLR2, that can activate in the same concentration range as LPS and flagellin, are bacterial lipopeptides and lipoproteins. Other described ligands activate TLR2 only at high concentrations and are mostly purified from bacterial cell walls that contain high amounts of lipoproteins [14]. *Staphylococcus aureus* (*S. aureus*) mutants deficient in lipoprotein maturation provide evidence that lipoproteins are the only ligand for TLR2 in staphylococci [15]. This suggests that the previous postulated ligands peptidoglycan and lipoteichoic acid (LTA) of *S. aureus* were contaminated with highly active natural lipoproteins or lipopeptides [14,16]. Although a co-crystal consisting of a TLR2 monomer together with LTA of *S. pneumoniae* was solved, dimerization of TLR2 together with TLR1 or TLR6 for LTA was not observed, in contrast to the co-crystal of the synthetic lipopeptide Pam2Cys with TLR2 [17]. TLR2 dimerization is necessary for proper activation and therefore TLR2-LTA interaction alone does not prove agonistic properties of LTA for TLR2. In a murine sepsis model *S. aureus* lacking lipoproteins is more virulent compared to wild-type [15]. Lipoprotein mutants fail to activate TLR2, resulting in uncontrolled growth of *S. aureus*. In contrast, in a similar model the absence of lipoproteins reduced the survival of *S. aureus* [18]. In this model, uptake of iron/nutrients via lipoproteins is essential for *S. aureus*, and probably beneficial over the cost of inducing a strong innate immune response via TLR2. Differences in the application route or the generation of the *S. aureus* lipoprotein mutant may explain the discrepancy between both studies. Lipid modifications that are present in a

wide variety of bacterial proteins serves as a powerful target of the innate immune system to control different invading bacteria. Since these modifications are only present in bacteria and at the same time essential for bacterial fitness and pathogenicity [9].

Proteins and Peptides

Proteins are conserved through all domains of life as proposed by Woese et al. in 1990 [19], and are therefore no candidates for recognition within the context of innate immunity. Exceptions to that are those proteins that have specific modifications that are only found in bacteria, such as formylation or the presence of D-amino acids. Furthermore proteins that are conserved but restricted to the bacterial kingdom can be exceptions too (flagellin).

Formyl-peptides

Bacteria synthesize proteins in a different way than eukaryotes and archaea. Translation always starts with a methionine residue that can be removed during protein maturation. Archaeic and eukaryotic proteins are synthesized with an unmodified primairy methionine residue, bacterial proteins contain a formylated methionine at the amino terminal end. This modification is the result of the action of the highly conserved bacterial enzyme: methionyl-tRNA-formyltransferase. The formyl group is typically removed by peptide deformylases upon protein maturation. However, the activity of the deformylases is not waterproof, and therefore growing bacteria are surrounded by a gradient of N-formylated proteins and peptides. The percentage of secreted formylated proteins can be quite substantial.

The prototype N-formyl-peptide, fMLP (better: fMLF or fMet-Leu-Phe) induces and potentiates chemotaxis, phagocytosis, and the generation of reactive oxygen species in both neutrophils and monocytes. Formylated peptides act on receptors belonging to the G-protein coupled receptors (GPCR) family: the formyl peptide receptor (FPR or FPR1) and its homologue FPR-like-1 (FPRL-1 or FPR2). FPR1 binds fMLP with high affinity, while FRP2 has low affinity for fMLP. Initially, FPR expression was demonstrated in monocytes, neutrophils, microglial and dendritic cells but it has also been described in non-hematopoietic cells and tissues [20,21].

Bacteria are not the only source of formylated peptides; mitochondria of eukaryotic cells, since they are of bacterial origin, also use N-formylmethionine as initiators of protein synthesis. These formylated proteins are released upon necrosis of cells and are a major cause of inflammatory reactions [22].

Dürr et al. [23] demonstrated that formylated peptides are indeed the major chemoattractants in *S. aureus* supernates. However, disruption of the formyl methionine transferase in this bacterium demonstrated that formyl peptides are not the only chemoattractants that are secreted. As with formylated peptides, the activity of the yet unknown chemoattractant molecules was mediated through GPCR signaling. A recent study by Wang et al. [24] showed that several phenol-soluble modulins can indeed prime neutrophils. Phenol-soluble modulins provoke neutrophil chemotaxis and induce calcium mobilization upon stimulation, suggesting involvement of GPCRs. Enhanced expression of phenol-soluble modulins in community-acquired methicillin-resistant *S. aureus* (CA-MRSA) is suggested to contribute to its virulence. Recently, we demonstrated that the human FPR2, which has previously been implicated in control of endogenous inflammatory processes, senses PSMs at nanomolar concentrations and initiates proinflammatory neutrophil responses to staphylococci, comparable to the mechanism by which FPR1 senses formylated peptides. However the

equivalent of PSMs in other bacterial species has yet to be determined [25].

Flagellin

Flagellated bacteria can swim to chemotactic or thermotactic gradients using their flagellum. The flagellar motor can either rotate counterclockwise, for a run, or clockwise, for tumbling, to change swimming direction [26]. Besides the motor, a flagellum consists of a hook and a long filament composed of polymerized flagellin, which serves as a propeller. Flagella consists of 11 protofilaments composed of flagellin monomers, that wrap together for the formation of the flagellar filament [27]. Flagellin consists of domains D0-D1 that form the flagellar filament tube and are highly conserved, and domains D2-D3 that stick out of the filament and are highly variable in sequence and length (from almost absent until one thousand residues). Sequence comparison of various flagellated bacteria revealed that the first 140 amino acids of the N-terminus and the last 90 amino acids of the C-terminus are highly conserved. This conserved part of flagellin is essential for proper filament assembly and motility, and forms the inner core of the flagellum. Therefore, recognition of this domain, that is conserved among most flagellated bacteria, serves as a good target for the innate immune system. Both mammals via TLR5 and plants via flagellin-sensitive 2 (FLS2) recognize the conserved domain of flagellin. This domain is normally hidden within the flagellum due to the variable part of the adjacent flagellin monomer (Fig. 5). Therefore, only monomeric flagellin is recognized by both receptors. Release of monomeric flagellin in the surroundings of flagellated bacteria is probably caused by degradation of flagella or dead bacteria. Given the fact that a flagellum consists of very large numbers of flagellin molecules (20,000), only

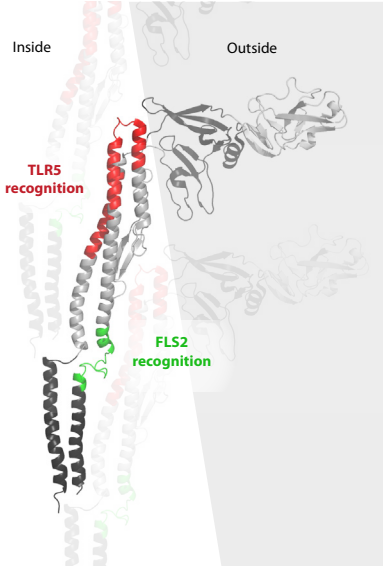

Figure 5. TLR5 and FLS2 recognition domains within flagellin
The monomeric form of flagellin consists of a conserved part (white area) and a variable part (grey area). Only the variable part is exposed in the polymeric flagellin form as present in flagella, the conserved part is shielded by the conserved domains of the two neighboring flagellin subunits. The flagellin domains recognized by FLS2 and TLR5 are depicted within the figure.

a small percentage of flagellin has to be released in its monomeric form to achieve potent activation of the innate immune system via flagellin sensing receptors.
In mammals, TLR5 is the only known TLR that has a ligand of protein origin. Flagellin triggers TLR5 in the picomolar range, the potency of flagellin differs between bacterial species (range 1-100 pM). Furthermore, there are difference in response of mouse and human TLR5 to flagellin from different bacterial species [28]. One amino acid change in the flagellin recognition site of TLR5 is sufficient for this discrimination. The structure of TLR5 is still not solved, only is silico predictions [29] and TLR5 mutational studies are available [30]. The conserved N- and C-terminal domain of flagellin are both essential in TLR5 activation [31]. Mutation of these conserved domains prevent activation via TLR5, however also severely impairs bacterial motility. These residues are located at the convex surface of flagellin monomers and essential for the interaction with other flagellin monomers to form flagellar filaments (Fig. 5).
Plants have a similar detection system for flagellated bacteria, they detect a linear part of 22 amino acids within the conserved N-terminal part of flagellin via FLS2 (Fig. 5) [32]. This epitope is completely different from the part that is recognized by TLR5. The sensitivity of whole plants to flagellin is much lower compared to plants cells. As for TLR5, FLS2 recognizes a part of flagellin, which is essential for proper flagellin assembly and motility.

Elongation factor tu (EF-tu)

The highly conserved cytosolic protein elongation factor Tu (EF-Tu) triggers plant defense systems via the elongation factor receptor (EFR). EF-Tu plays an essential role in the transfer of aminoacylated transfer RNA to the messenger RNA:ribosome complex, which is necessary for protein synthesis [33]. Elongation factor Tu is one of the most abundant and highly conserved proteins of bacteria [34]. This receptor recognizes a linear part of EF-Tu, which can be mimicked by a 18 amino acid long peptide [35]. This perception system recognizes sequences containing the motif: acetyl-xKxKFxR. This motif is characteristic and highly specific for the N-terminus of bacterial elongation factors. *Arabidopsis* cells respond to the N-terminal part of EF-Tu in low nanomolar concentrations, in contrast to whole plants, which respond to higher concentrations. Elongation factor is one the most slowly evolving proteins [36], homology can be found between all bacterial, but also between eukaryotic elongation factors. Although plant and bacterial elongation factors share homology, *Arabidopsis* EF-tu is not sensed via EFR [35]. Elongation factor is a highly abundant protein (5-9% of the total bacterial cell protein content) and the protein is present in the secretome of different bacteria [37]. The function of EFR is illustrated by the fact that *Arabidopsis* mutants that lack the gene encoding the EFR are more susceptible to *Agrobacterium* infection, as compared to control plants [37].
The elongation factor receptor triggers a common set of signaling and defense responses as observed for FLS2-flagellin. Striking is the difference in the origin of bacterial ligands between mammals and plants. In mammals, the activators are glycolipids, sugars, proteins or peptides, whereas the two identified receptors of Arabidopsis, EFR and FLS2, both recognize a linear epitope of about 20 residues of a conserved bacterial protein.

Carbohydrates

A vast number of cellular receptors and soluble lectins or lectin-like molecules have been identified to interact with carbohydrates and carbohydrate motifs in order to facilitate the

distinction between non-self and self (Fig. 6).
The collagenous lectins or collectins (e.g. mannose-binding lectin (MBL)), surfactant proteins SP-A and SP-D and conglutinin), and the ficolins are soluble pattern-recognition receptors. They are present in human plasma or in the lung, and act as indirect recognition molecules. Some activate the complement pathway (MBL and the ficolins), others interact with cellular receptors to provoke inflammatory events. MBL recognizes carbohydrate structure of many different bacteria [38]. Ficolins are a family of oligomeric proteins consisting of an N-terminal collagen-like domain and a C-terminal globular fibrinogen-like domain. Ficolins specifically recognize N-acetyl compounds such as N-acetylglucosamine, components of bacterial and fungal cell walls, and certain bacteria. As for MBL, ficolins are in complex with MBL-associated serine proteases (MASPs). These MASPs activate the complement system through the lectin pathway. This triggers phagocytosis and bacterial lysis. In humans, three ficolins (L-, M- and H-ficolins) have been identified, which exhibit differences in ligand binding and bacterial recognition, suggesting specific roles for each ficolin.
Binding of host lectins to the glycans of foreign pathogens is involved in the innate immunity. C-type lectins include the mannose receptor and the well know dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Receptors that have a clear function in cellular activation. Of special interest are the RegIII proteins, secreted C-type lectins that kill Gram-positive bacteria and play a vital role in antimicrobial protection of the mammalian gut. RegIII proteins bind their bacterial targets via interactions with cell wall peptidoglycan but lack the canonical sequences that support calcium-

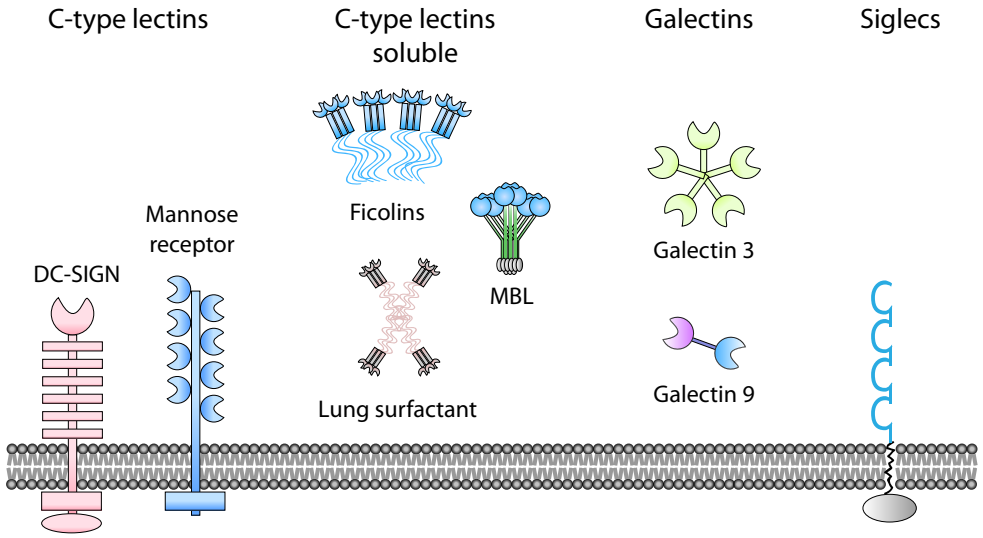

Figure 6. Recognition of bacterial carbohydrates and lectins
Overview of carbohydrate recognition molecules of the innate immune system. The family of C-type lectins consists of soluble pattern-recognition molecules, such as mannose binding lectin that can activate the complement system and receptors like DC-SIGN involved in cellular activation. The soluble galectins bind to N-acetyllactosamine-containing glycans on bacterial surfaces. Galectins interact with sialylated glycoconjugates and can trigger cellular activation.

dependent carbohydrate binding in other C-type lectins [39]. Siglecs (sialic acid binding Ig-like lectins) are characterized by an N-terminal Ig domain that mediates sialic acid binding, They are present on a variety of white blood cells (macrophages, B-cells) and play a clear role in cellular activation as well as deactivation. They recognize sialylated glycoconjugates [40]. Galectins were first characterized in recognition of ('self') carbohydrate ligands in embryogenesis, development and immune regulation. However, now galectins have been shown to bind *N*-acetyllactosamine-containing glycans on the surface of potentially pathogenic microorganisms, and function as recognition and effector factors in innate immunity [41]. Galectins can interact directly with bacterial surface glycans on both Gram-positive, and Gram-negative bacteria, fungi, and parasites.

Specificity of these lectins for pathogens is determined by density-dependent glycan binding and pattern-recognition and not by the nature of the monosaccharides involved. In many cases, these monosaccharides form a low affinity epitope. Host lectins may bind to such "weak epitopes" due to large increases in the affinity of the lectin for polyvalent displays of the glycan. Two mechanisms are identified for lectin binding to density dependent glycans: (1) binding and internal diffusion of lectins such as galectins among a dense population of glycans, and (2) binding of multiple CRD domains of lectins such as soluble C-type lectins to clustered glycan epitopes or a combination of both.

A special note on peptidoglycan recognition. TLR2 was long seen as the receptor for peptidoglycan, but the observed TLR2 activity was probably due a contaminating lipoprotein [13]. Peptidoglycan recognition by peptidoglycan recognition proteins (PGRPs) involves moderate- to high-affinity interactions with the peptide moiety, allowing discrimination between Gram-positive and Gram-negative bacteria and the initiation of immune responses specific for either group. PGRPs are innate immunity proteins that are conserved from insects to mammals, they recognize bacterial peptidoglycan, and function in antibacterial immunity and inflammation. Mammals have four PGRPs, some of which are also directly bactericidal due to their amidase activity. The PGRPs likely play a role both in antibacterial defenses and several inflammatory diseases.

Bacterial RNA and DNA

DNA and RNA are widely conserved among every living organism on earth. It is hard to imagine that these compounds could form the basis of molecular discrimination between host and pathogen. However some receptors mange to discriminate between self and non-self DNA and RNA. For bacterial RNA it is reported that it specifically can activate human dendritic cells and that besides activation of TLR7 and inflammasomes, bacterial RNA activates additional cytosolic receptors similarly as has been reported for recognition of bacterial DNA. Bacterial DNA is recognized by TLR9 and it was shown that the immunostimulatory activity of bacterial DNA was due to the presence of unmethylated CpG dinucleotides. The activity is specific for bacterial DNA, as mammalian genomic DNA is completely inactive in this respect. Bacterial DNA activates mainly dendritic cells and subpopulations, thereby inducing an intense interferon and IL-12 response. Therefore, CpG DNA represents a new and promising class of adjuvant for vaccination.

Bacteria

Commensal bacteria colonize mucosal membranes and exceed the number of cells in our body with a factor of ten. They play an important role in the development of the immune system and in defense against invading pathogens. Some bacteria manage to bypass the physical barriers and the innate immune system of the host and cause infections. These pathogens are equipped with a wide array of effector mechanisms such as secretion of small proteins, proteases and formation of capsules to evade detection and eradication by the innate immune system.

Pseudomonas

The genus *Pseudomonas* belongs to the class of gammaproteobacteria. Pseudomonads are Gram-negative rod-shaped bacteria that are present in soil and water, and contain a polar flagellum to move quickly through aqueous environments. *Pseudomonas aeruginosa* (*P. aeruginosa*) is an emerging opportunistic pathogen that causes severe infections in cystic fibrosis and immunocompromised patients in contrast to other *Pseudomonas* species. Cystic fibrosis patients suffer from chronic infections and cannot clear this bacterium from their airways despite of extensive antibiotic treatment [42]. Treatment of *P. aeruginosa* is hampered by chromosomically encoded and acquired resistance genes via mobile elements. *P. aeruginosa* can easily adopt to its environment, as demonstrated by isolates from the airways of cystic fibrosis patients. These strains contain various mutations in regulatory genes and inactivate the DNA mismatch repair system, leading to hypermutator phenotypes [43]. In addition, these strains produce very high levels of the carbohydrate alginate in comparison to normal laboratory strains [44]. Alginate causes the typical mucoid phenotype of *P. aeruginosa* strains isolated from cystic fibrosis patients. In nature, *P. aeruginosa* is found as free floating (planktonic growth) motile bacterium in aqueous environments or in dense non-motile communities known as biofilms. These biofilms enhance the resistance of the bacterium to hostile environments. In patients, biofilm formation impairs antimicrobial treatment due to impaired penetration of antibiotics into the biofilm, enhanced persister cell formation (slow dividing bacteria that are less sensitive to antibiotics) and differential physiological states of biofilm subpopulations [45]. *P. aeruginosa* biofilm formation is also observed in the lungs of cystic fibrosis patients [46].
In plants, *Pseudomonas* species colonize the leaf surface without causing disease [47]. *Pseudomonas* cannot directly penetrate through the leaf epidermis [48]. Entrance occurs through natural openings in the leaves, such as stomata or wounds. This triggers defense responses to restrict bacterial proliferation via recognition of conserved molecular patterns such as flagellin and LPS [49]. *P. syringae* is a plant pathogen that infect several plants and causes crop losses in agriculture. The host-pathogen interaction of *P. syringae* and the model plant *Arabidopsis thaliana* has been intensively studied [47]. A major pathogenesis mechanism of *P. syringae* is injection of effector proteins via a type III secretion system. These virulence factors interfere with plant immunity, for example the recognition of flagellin via the FLS2 in *Arabidopsis* [50].

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium that frequently causes infections in both the hospital and community. Infections with *S. aureus* occurs at diverse

sites of the body and range from mild skin infections to life-threatening diseases such as sepsis. The most frequently colonized surface in humans is the nose, around 20% of the population are persistent nasal carriers [51]. Increasing resistance to different antibiotics impairs treatment of *S. aureus* in the clinic [52]. *S. aureus* has a wide arsenal of virulence factors that contribute to bacteria pathogenesis. Expression of surface proteins that interact with extracellular matrix proteins of the host contribute to bacterial adherence [53]. After adherence, secretion of numerous proteases facilitate invasion of host tissue by *S. aureus*. The expression of virulence factors is regulated by the accessory gene regulator (agr) quorum sensing system. Expression of virulence factors is coordinated to reduce unnecessary metabolic demands and is essential for bacterial pathogenesis.

Immune evasion

To survive in the human host, bacteria have developed many different strategies to escape from the innate immune response, including the expression of an extracellular capsule, and 'hiding' within host cells, either in the vacuole or in the cytoplasm. In the past few years it has become clear that, in addition to these mechanisms, bacteria can escape recognition by secretion of small proteins that interfere with activation of the innate immune system [54]. Staphylococci secrete several of these molecules to protect itself from destruction by the complement system and phagocytes. *P. aeruginosa* secretes several proteases that degrade and inactivate components of the innate immune system. Another bacterial strategy is modification of conserved structures that are recognized by pattern recognition receptors. This will reduce bacterial fitness (e.g. impaired motility), but will help to be undetectable for the innate immune system.

Ligand manipulation

Even at low concentrations lipopolysaccharide triggers strong inflammatory responses. Several bacteria manipulate the number and composition of acyl chains of lipid A upon environmental triggers. *P. aeruginosa* manipulates its LPS during chronic infection of the cystic fibrosis lung [55]. Modification depends on the disease stage of the cystic fibrosis patient and is linked with differences in biological activity of LPS [56,57]. The normally penta-acylated form of LPS produced by *P. aeruginosa* grown under laboratory conditions is partly substituted to hexa- and hepta-acylated LPS in cystic fibrosis patients. These modifications increase the stimulatory activity of LPS with more than 100-fold [58]. The hexa-acylated form of lipid A increases the resistance of *P. aeruginosa* to antimicrobial peptides. Bacterial hosts differ in their response to lipid A modifications of *P. aeruginosa*, murine TLR4 react in the same way, while human TLR4 can discriminate between these lipid A forms. This suggests that not only bacteria but also their hosts have adapted to recognize different forms of lipid A.

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) modifies its LPS via the PhoP-PhoQ modification system [59]. This system responds to environmental triggers, such as exposure to antimicrobial peptides, via PhoQ, which phosphorylates PhoP. This induces gene expression of PagL/PagP, involved in deacylation and palmitoylation of LPS, respectively. LPS isolated form *E. coli* heterologously expressing PagL and/or PagP showed a 30-100 fold lower activation of NF-κB compared to unmodified LPS [60]. In this way, *S.* Typhimurium can

reduce the TLR4 stimulatory activity of its LPS upon environmental triggers.
The lipid A composition of *Yersinia pestis* depends on temperature. In nature, *Y. pestis* is transmitted via the bite of infected fleas to mammalian hosts causing bubonic plague. Lipid A isolated from *Y. pestis* grown at 21-27°C (fleas temperature) is a potent TLR4 activator, whereas lipid A isolated when grown at 37°C (mammalian temperature) has poor immunostimulatory activity. Analysis of the acylation of lipid A revealed that the number of acyl chains changed from six at lower to four at higher temperatures. Furthermore, LPS that is produced at higher temperatures can antagonize hexa-acylated LPS that is produced at lower temperatures [61]. In this way, *Y. pestis* prevents activation of TLR4 in mammalian hosts.
The evolutionary pressure to preserve the part of flagellin necessary for proper filament assembly is illustrated by the conservation of flagellin domains that interact with other flagellin molecules (Fig. 5). However, some bacteria managed to escape recognition via TLR5 by mutation of this conserved part of flagellin that is essential for filament assembly and motility. Flagellin from the highly motile *α* and *ε proteobacteria, Helicobacter pylori* and *Campylobacter jejuni* [62] does not activate TLR5. Comparison of the amino acid sequences of flagellin from these non-stimulatory with TLR5-stimulatory bacteria revealed differences within the conserved D1 domain. Replacement of these residues of *S.* Typhimurium flagellin with the corresponding domain of *H. pylori* results in loss of motility and TLR5 recognition [62]. Beside mutations in the TLR5 recognition domain, flagellin from *α* and *ε proteobacteria*, contains compensatory mutations to maintain proper flagellar filament assembly and motility.

Immune evasion by secreted proteins of *S. aureus*

Staphylococci evade innate immunity in a different way; they secrete a vast array of immunomodulatory proteins that hinder the molecular recognition events that have been described above. First, different molecules are secreted that interfere with opsonins that are already deposited on the surface of the bacterium. Protein A, staphylococcal binder of immunoglobulins (Sbi) and staphylococcal superantigen-like protein 10 (SSL10) all bind IgG molecules that are deposited on the surface of the bacterium [63,64]. In this way these molecules, independently, interfere with activation of the classical pathway of complement, and directly interfere with Fc receptor-mediated phagocytosis. Staphylokinase (SAK) converts surface bound plasminogen into plasmin. This surface located plasmin cleaves IgG and the complement factor C3b that are already present on the surface of bacteria. In this way, SAK acts as an opsonin remover, and is therefore antiphagocytic [65]. Furthermore, SAK can also cleave antimicrobial peptides and thus helps to evade innate immunity in different ways [66].
Next to the opsonin removers staphylococci have evolved a set of proteins that inhibit complement activation, and thereby block all inflammatory signals such as ficolin and MBL-mediated recognition pathways. These molecules prevent opsonization, but since most of them also prevent the generation of C5a (more downstream in the complement activation cascade), they are also inhibitors of cellular activation. Staphylococcal complement inhibitor (SCIN) and its homologues SCIN-B and SCIN-C bind and inhibit the classical, lectin, and alternative pathway C3 convertase. SCIN blocks, stabilizes and dimerizes the alternative pathway C3 convertase. In this way, SCIN, SCIN-B, and SCIN-C interfere primarily with C3b deposition and thereby inhibit phagocytosis [67,68]. Sbi, next to its function as an IgG

binding molecule also inhibits complement activation by binding fH in combination with C3b. The dual action on C3b and IgG decreases phagocytosis [69]. The extracellular fibrinogen-binding protein (Efb) and the homologous extracellular complement binding protein (Ecb) bind to C3b and C3d. Functionally this binding of Efb and Ecb blocks the alternative pathway C3 convertase and down-modulates all C5 convertases, resulting especially in diminished C5a generation, thereby preventing neutrophil activation and neutrophil migration towards the site of infection [67]. Aureolysin, a secreted metalloprotease cleaves C3 in such a way that it opens up the molecule for further cleavage into non functional C3 fragments, thereby drastically diminishing C3b deposition [70]. SSL7 binds to complement factor C5, and thereby also inhibits C5 conversion into C5a as was demonstrated both in vitro as well as in vivo [71,72]. The chemotaxis inhibitory protein of staphylococci (CHIPS) acts directly on the C5a receptor, it binds to the C5a binding site. In this way, CHIPS blocks the action of C5a, and represents an alternative strategy to interfere with cellular migration and activation [73].
Alternative strategies to keep neutrophils at a distance are employed by SSL5. It acts on different chemokines and chemokine receptors to prevent activation of neutrophils by several chemokines [74], and binds P-selectin glycoprotein Ligand-1 (PSGL-1) on neutrophils [75]. Intracellular adhesion molecule-1 (ICAM-1) is bound and inhibited by extracellular adherence protein (Eap) [76]. This blocks the final molecular adherence step in primary rolling and transmigration events in the blood stream near the site of infection.
As outlined above formylated peptides provide a bacteria-specific broad signature that is sensed by formylpeptide receptors. *S. aureus* has evolved at least two antagonists for the FPR. Both chemotaxis inhibitory protein of *S. aureus* (CHIPS) and FPRL-1 inhibiting protein-like (FLIPr-like) inhibit the FPR, and thereby avoid recognition via this unique recognition pathway [77]. FLIPr and FLIPr-like inhibit FPRL-1 and thus evades recognition of the phenol soluble modulins that are secreted by staphylococci. All these molecules together inhibit migration towards the site of infection by the very first chemoattractants present upon infection. In addition, cellular activation, important for co-signaling events during phagocytosis is inhibited.

Proteases and immune evasion by *P. aeruginosa*

Proteases cleave peptide bonds and can be divided in six different groups, serine-, threonine-, cysteine-, aspartate-, metallo- and glutamic acid proteases. Some proteases recognize a specific recognition site present in one (e.g. complement convertases) or only a few proteins, while others cleave a peptide bond between two specific amino acids. Metalloproteases have very diverse functions in biological processes, such as intestinal absorption of nutrients, embryonal development, and metabolism of antibiotics [78]. The active site of metalloproteases is characterized by a divalent metal ion that is coordinated by three amino acids. Proteolytic activity can be inhibited by the divalent metal chelator EDTA. The metal ion in the active site is usually zinc, however in some cases cobalt, nickel or manganese. The amino acids histidine, glutamate, asparagine or lysine coordinate the metal ion in the active site of the protease. In the active site a water molecule bound to the catalytic zinc ion is essential for hydrolysis of the peptide bond of the substrate. The carbonyl group of the substrate interacts with the catalytic zinc ion and displaces the water molecule. Followed by a nucleophilic attack of the water molecule on the carbonyl carbon of the peptide bond to be cleaved (scissile bond).
Metalloproteases can be divided in several classes depending on their active site. Most

metalloproteases contain the HEXXH catalytic metal binding motif. The subclass of metzincins all contain the zinc binding motif HEXXHXXGXXH/D. In these proteases two histidines (and a third histidine or aspartate coordinate the zinc ion in the active site, and they all contain a methionine-containing 1,4 turn in a similar conformation [78]. Alkaline protease of *P. aeruginosa* belongs to the serralysins, a subdivision of the metzincin family. Serralysins are present in other (pathogenic) Gram-negative bacteria like *Yersinia pestis*, *Serratia marcecens,* and *Erwinia chrysanthemi* [79].
Alkaline protease degrades different proteins of the immune system, like complement components [80], immunoglobulins and cytokines [81]. Production of alkaline protease can be detected in cystic fibrosis sputum and has been associated with bacterial virulence [82,83]. *P. aeruginosa* secretes several other zinc metalloproteases like elastase, also known as pseudolysin, and LasA, also known as staphylolysin. Both proteases show activity for elastin [84] and belong to another protease family as alkaline protease. LasA cleaves peptide bonds following Gly-Gly and can cause lysis of *Staphylococcus aureus* by degradation of peptidoglycan [85]. *P. aeruginosa* elastase degrades antimicrobial peptides, chemokines, cytokines [81] and the phagocytic receptor uPAR [86]. Furthermore, elastase is involved in *P. aeruginosa* virulence [87].

Outline of the thesis

The innate immune system is very efficient in destroying invading bacteria by recognition of evolutionary conserved microbial structures. In this way all bacteria, viruses and fungi can be recognized by a limited number of innate recognition molecules. Bacteria have developed various strategies to evade recognition by the innate immune system, and thereby escape killing to become a successful pathogen. In the past decade a wide variety of bacterial immune evasion molecules have been identified. These small secreted proteins disarm various branches of the innate immune system by blocking crucial steps in recognition and activation cascades.
Members of the Toll-like receptor family recognize evolutionary conserved microbial structures and trigger inflammatory responses. Bacteria struggle to maintain these structures that are essential for their fitness and at the same time initiate activation and killing via the innate immune system. However, some bacteria have succeeded to manipulate their TLR ligands in such a way that they evade recognition without major functional consequences. This evolutionary pressure to manipulate essential molecules indicate that escape of TLR activation is crucial for bacterial survival. We hypothesize that bacteria have developed inhibitors that interfere with Toll-like receptor recognition as observed for other parts of the innate immune system. The aim of this thesis is to identify secreted bacterial proteins directed against TLR1, 2, 4, 5 and 6, as well as, LBP, CD14 and MD-2.
Identification of TLR inhibitors will help to understand bacterial pathogenesis. Although TLRs defend the host against invading pathogens, over-activation is the basis of several inflammatory diseases. The mechanism of action and the target sites of TLR inhibitors designed by bacteria can be of interest for development of future anti-inflammatory molecules.

References

1. Gasque P (2004) Complement: a unique innate immune sensor for danger signals. Mol Immunol 41: 1089-1098.
2. Roos D, van Bruggen R, Meischl C (2003) Oxidative killing of microbes by neutrophils. Microbes Infect 5: 1307-1315.
3. Mayer-Scholl A, Averhoff P, Zychlinsky A (2004) How do neutrophils and pathogens interact? Curr Opin Microbiol 7: 62-66.
4. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, et al. (2009) The role of the anaphylatoxins in health and disease. Mol Immunol 46: 2753-2766.
5. Trent MS, Stead CM, Tran AX, Hankins JV (2006) Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res 12: 205-223.
6. Bryant CE, Spring DR, Gangloff M, Gay NJ (2010) The molecular basis of the host response to lipopolysaccharide. NatRevMicrobiol 8: 8-14.
7. Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI (2002) Human Toll-like receptor 4 recognizes host-specific LPS modifications. NatImmunol 3: 354-359.
8. Park BS, Song DH, Kim HM, Choi BS, Lee H, et al. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458: 1191-1195.
9. Schmaler M, Jann NJ, Gotz F, Landmann R (2010) Staphylococcal lipoproteins and their role in bacterial survival in mice. Int J Med Microbiol 300: 155-160.
10. Schenk M, Belisle JT, Modlin RL (2009) TLR2 looks at lipoproteins. Immunity 31: 847-849.
11. Babu MM, Priya ML, Selvan AT, Madera M, Gough J, et al. (2006) A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. JBacteriol 188: 2761-2773.
12. Shimizu T, Kida Y, Kuwano K (2007) Triacylated lipoproteins derived from Mycoplasma pneumoniae activate nuclear factor-kappaB through toll-like receptors 1 and 2. Immunology 121: 473-483.
13. Buwitt-Beckmann U, Heine H, Wiesmuller KH, Jung G, Brock R, et al. (2005) Toll-like receptor 6-independent signaling by diacylated lipopeptides. EurJImmunol 35: 282-289.
14. Zahringer U, Lindner B, Inamura S, Heine H, Alexander C (2008) TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparant broad specificity. Immunobiology 213: 205-224.
15. Bubeck Wardenburg J, Williams WA, Missiakas D (2006) Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins. Proc Natl Acad Sci U S A 103: 13831-13836.
16. Travassos LH, Girardin SE, Philpott DJ, Blanot D, Nahori MA, et al. (2004) Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep 5: 1000-1006.
17. Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, et al. (2009) Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity 31: 873-884.
18. Schmaler M, Jann NJ, Ferracin F, Landolt LZ, Biswas L, et al. (2009) Lipoproteins in Staphylococcus aureus mediate inflammation by TLR2 and iron-dependent growth in vivo. J Immunol 182: 7110-7118.
19. Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U S A 87: 4576-4579.
20. Le Y, Gong W, Tiffany HL, Tumanov A, Nedospasov S, et al. (2001) Amyloid (beta)42 activates a G-protein-coupled chemoattractant receptor, FPR-like-1. J Neurosci 21: RC123.
21. Migeotte I, Communi D, Parmentier M (2006) Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine Growth Factor Rev 17: 501-519.
22. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, et al. (2010) Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature 464: 104-107.
23. Durr MC, Kristian SA, Otto M, Matteoli G, Margolis PS, et al. (2006) Neutrophil chemotaxis by pathogen-associated molecular patterns--formylated peptides are crucial but not the sole neutrophil attractants produced by

Staphylococcus aureus. Cell Microbiol 8: 207-217.
24. Wang R, Braughton KR, Kretschmer D, Bach TH, Queck SY, et al. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1510-1514.
25. Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, et al. (2010) Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. Cell Host Microbe 7: 463-473.
26. Maki-Yonekura S, Yonekura K, Namba K (2010) Conformational change of flagellin for polymorphic supercoiling of the flagellar filament. Nat Struct Mol Biol 17: 417-422.
27. Ramos HC, Rumbo M, Sirard JC (2004) Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. Trends Microbiol 12: 509-517.
28. Andersen-Nissen E, Smith KD, Bonneau R, Strong RK, Aderem A (2007) A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin. JExpMed 204: 393-403.
29. Jacchieri SG, Torquato R, Brentani RR (2003) Structural study of binding of flagellin by Toll-like receptor 5. JBacteriol 185: 4243-4247.
30. Mizel SB, West AP, Hantgan RR (2003) Identification of a sequence in human toll-like receptor 5 required for the binding of Gram-negative flagellin. J Biol Chem 278: 23624-23629.
31. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, et al. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 4: 1247-1253.
32. Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J 18: 277-284.
33. Krab IM, Parmeggiani A (1998) EF-Tu, a GTPase odyssey. Biochim Biophys Acta 1443: 1-22.
34. Caldon CE, Yoong P, March PE (2001) Evolution of a molecular switch: universal bacterial GTPases regulate ribosome function. Mol Microbiol 41: 289-297.
35. Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, et al. (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell 16: 3496-3507.
36. Lathe WC, 3rd, Bork P (2001) Evolution of tuf genes: ancient duplication, differential loss and gene conversion. FEBS Lett 502: 113-116.
37. Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, et al. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125: 749-760.
38. Eisen DP, Minchinton RM (2003) Impact of mannose-binding lectin on susceptibility to infectious diseases. Clin Infect Dis 37: 1496-1505.
39. Lehotzky RE, Partch CL, Mukherjee S, Cash HL, Goldman WE, et al. (2010) Molecular basis for peptidoglycan recognition by a bactericidal lectin. Proc Natl Acad Sci U S A 107: 7722-7727.
40. Crocker PR, Varki A (2001) Siglecs in the immune system. Immunology 103: 137-145.
41. Rabinovich GA, Toscano MA, Jackson SS, Vasta GR (2007) Functions of cell surface galectin-glycoprotein lattices. Curr Opin Struct Biol 17: 513-520.
42. Gibson RL, Burns JL, Ramsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med 168: 918-951.
43. Feliziani S, Lujan AM, Moyano AJ, Sola C, Bocco JL, et al. (2010) Mucoidy, quorum sensing, mismatch repair and antibiotic resistance in Pseudomonas aeruginosa from cystic fibrosis chronic airways infections. PLoS One 5.
44. Qiu D, Eisinger VM, Rowen DW, Yu HD (2007) Regulated proteolysis controls mucoid conversion in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 104: 8107-8112.
45. Harmsen M, Lappann M, Knochel S, Molin S (2010) Role of extracellular DNA during biofilm formation by Listeria monocytogenes. Appl Environ Microbiol 76: 2271-2279.
46. Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, et al. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762-764.
47. Gimenez-Ibanez S, Rathjen JP (2010) The case for the defense: plants versus Pseudomonas syringae. Microbes

Infect 12: 428-437.
48. Underwood W, Melotto M, He SY (2007) Role of plant stomata in bacterial invasion. Cell Microbiol 9: 1621-1629.
49. Melotto M, Underwood W, Koczan J, Nomura K, He SY (2006) Plant stomata function in innate immunity against bacterial invasion. Cell 126: 969-980.
50. Li X, Lin H, Zhang W, Zou Y, Zhang J, et al. (2005) Flagellin induces innate immunity in nonhost interactions that is suppressed by Pseudomonas syringae effectors. Proc Natl Acad Sci U S A 102: 12990-12995.
51. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, et al. (2005) The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis 5: 751-762.
52. de Lencastre H, Oliveira D, Tomasz A (2007) Antibiotic resistant Staphylococcus aureus: a paradigm of adaptive power. Curr Opin Microbiol 10: 428-435.
53. Gordon RJ, Lowy FD (2008) Pathogenesis of methicillin-resistant Staphylococcus aureus infection. Clin Infect Dis 46 Suppl 5: S350-359.
54. Rooijakkers SH, van Kessel KP, van Strijp JA (2005) Staphylococcal innate immune evasion. Trends Microbiol 13: 596-601.
55. Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, et al. (1999) Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas aeruginosa. Science 286: 1561-1565.
56. Cigana C, Curcuru L, Leone MR, Ierano T, Lore NI, et al. (2009) Pseudomonas aeruginosa exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. PLoS One 4: e8439.
57. Ernst RK, Adams KN, Moskowitz SM, Kraig GM, Kawasaki K, et al. (2006) The Pseudomonas aeruginosa lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. J Bacteriol 188: 191-201.
58. Ernst RK, Hajjar AM, Tsai JH, Moskowitz SM, Wilson CB, et al. (2003) Pseudomonas aeruginosa lipid A diversity and its recognition by Toll-like receptor 4. J Endotoxin Res 9: 395-400.
59. Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, et al. (1997) Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. Science 276: 250-253.
60. Kawasaki K, Ernst RK, Miller SI (2004) 3-O-deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of Salmonella typhimurium, modulates signaling through Toll-like receptor 4. J Biol Chem 279: 20044-20048.
61. Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, et al. (2006) Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. Nat Immunol 7: 1066-1073.
62. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, et al. (2005) Evasion of Toll-like receptor 5 by flagellated bacteria. ProcNatlAcadSciUSA 102: 9247-9252.
63. Itoh S, Hamada E, Kamoshida G, Yokoyama R, Takii T, et al. (2010) Staphylococcal superantigen-like protein 10 (SSL10) binds to human immunoglobulin G (IgG) and inhibits complement activation via the classical pathway. Mol Immunol 47: 932-938.
64. Atkins KL, Burman JD, Chamberlain ES, Cooper JE, Poutrel B, et al. (2008) S. aureus IgG-binding proteins SpA and Sbi: host specificity and mechanisms of immune complex formation. Mol Immunol 45: 1600-1611.
65. Rooijakkers SH, van Wamel WJ, Ruyken M, van Kessel KP, van Strijp JA (2005) Anti-opsonic properties of staphylokinase. Microbes Infect 7: 476-484.
66. Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J, et al. (2004) Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J Immunol 172: 1169-1176.
67. Jongerius I, Kohl J, Pandey MK, Ruyken M, van Kessel KP, et al. (2007) Staphylococcal complement evasion by various convertase-blocking molecules. J Exp Med 204: 2461-2471.
68. Rooijakkers SH, Ruyken M, Roos A, Daha MR, Presanis JS, et al. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol 6: 920-927.
69. Haupt K, Reuter M, van den Elsen J, Burman J, Halbich S, et al. (2008) The Staphylococcus aureus protein Sbi acts as a complement inhibitor and forms a tripartite complex with host complement Factor H and C3b. PLoS Pathog

4: e1000250.
70. Laarman AJ, Ruyken M, Malone CL, van Strijp JA, Horswill AR, et al. (2011) Staphylococcus aureus Metalloprotease Aureolysin Cleaves Complement C3 To Mediate Immune Evasion. J Immunol.
71. Bestebroer J, Aerts PC, Rooijakkers SH, Pandey MK, Kohl J, et al. (2010) Functional basis for complement evasion by staphylococcal superantigen-like 7. Cell Microbiol 12: 1506-1516.
72. Laursen NS, Gordon N, Hermans S, Lorenz N, Jackson N, et al. (2010) Structural basis for inhibition of complement C5 by the SSL7 protein from Staphylococcus aureus. Proc Natl Acad Sci U S A 107: 3681-3686.
73. de Haas CJ, Veldkamp KE, Peschel A, Weerkamp F, Van Wamel WJ, et al. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. J Exp Med 199: 687-695.
74. Bestebroer J, van Kessel KP, Azouagh H, Walenkamp AM, Boer IG, et al. (2009) Staphylococcal SSL5 inhibits leukocyte activation by chemokines and anaphylatoxins. Blood 113: 328-337.
75. Bestebroer J, Poppelier MJ, Ulfman LH, Lenting PJ, Denis CV, et al. (2007) Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 109: 2936-2943.
76. Chavakis T, Hussain M, Kanse SM, Peters G, Bretzel RG, et al. (2002) Staphylococcus aureus extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. Nat Med 8: 687-693.
77. Prat C, Haas PJ, Bestebroer J, de Haas CJ, van Strijp JA, et al. (2009) A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. J Immunol 183: 6569-6578.
78. Nagase H (2001) Metalloproteases. Curr Protoc Protein Sci Chapter 21: Unit 21 24.
79. Gomis-Ruth FX (2003) Structural aspects of the metzincin clan of metalloendopeptidases. Mol Biotechnol 24: 157-202.
80. Hong YQ, Ghebrehiwet B (1992) Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol 62: 133-138.
81. Parmely M, Gale A, Clabaugh M, Horvat R, Zhou WW (1990) Proteolytic inactivation of cytokines by Pseudomonas aeruginosa. Infect Immun 58: 3009-3014.
82. Jaffar-Bandjee MC, Lazdunski A, Bally M, Carrere J, Chazalette JP, et al. (1995) Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by Pseudomonas aeruginosa. J Clin Microbiol 33: 924-929.
83. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog 2: e56.
84. Toder DS, Gambello MJ, Iglewski BH (1991) Pseudomonas aeruginosa LasA: a second elastase under the transcriptional control of lasR. Mol Microbiol 5: 2003-2010.
85. Kessler E, Safrin M, Olson JC, Ohman DE (1993) Secreted LasA of Pseudomonas aeruginosa is a staphylolytic protease. J Biol Chem 268: 7503-7508.
86. Leduc D, Beaufort N, de Bentzmann S, Rousselle JC, Namane A, et al. (2007) The Pseudomonas aeruginosa LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain-specific endoproteolysis. Infect Immun 75: 3848-3858.
87. Doring G, Dalhoff A, Vogel O, Brunner H, Droge U, et al. (1984) In vivo activity of proteases of Pseudomonas aeruginosa in a rat model. J Infect Dis 149: 532-537.

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Pseudomonas Evades Immune Recognition of Flagellin in Both Mammals and Plants

Bart W. Bardoel[a], Sjoerd van der Ent[b,c], Michiel J.C. Pel[b,c], Jan Tommassen[d], Corné M.J. Pieterse[b.c], Kok P.M. van Kessel[a], and Jos A.G. van Strijp[a]

[a] Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands.
[b] Plant-Microbe Interactions, Institute of Environmental Biology, Faculty of Science, Utrecht University, The Netherlands.
[c] Centre for BioSystems Genomics, Wageningen, The Netherlands
[d] Department of Molecular Microbiology, Institute of Biomembranes, Utrecht University, The Netherlands.

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Abstract

The building blocks of bacterial flagella, flagellin monomers, are potent stimulators of host innate immune systems. Recognition of flagellin monomers occurs by flagellin-specific pattern-recognition receptors, such as Toll-like receptor 5 (TLR5) in mammals and flagellin-sensitive 2 (FLS2) in plants. Activation of these immune systems via flagellin leads eventually to elimination of the bacterium from the host. In order to prevent immune activation and thus favor survival in the host, bacteria secrete many proteins that hamper such recognition. In our search for Toll like receptor (TLR) antagonists, we screened bacterial supernatants and identified alkaline protease (AprA) of *Pseudomonas aeruginosa* as a TLR5 signaling inhibitor as evidenced by a marked reduction in IL-8 production and NF-κB activation. AprA effectively degrades the TLR5 ligand monomeric flagellin, while polymeric flagellin (involved in bacterial motility) and TLR5 itself resist degradation. The natural occurring alkaline protease inhibitor AprI of *P. aeruginosa* blocked flagellin degradation by AprA. *P. aeruginosa aprA* mutants induced an over 100-fold enhanced activation of TLR5 signaling, because they fail to degrade excess monomeric flagellin in their environment. Interestingly, AprA also prevents flagellin-mediated immune responses (such as growth inhibition and callose deposition) in *Arabidopsis thaliana* plants. This was due to decreased activation of the receptor FLS2 and clearly demonstrated by delayed stomatal closure with live bacteria in plants. Thus, by degrading the ligand for TLR5 and FLS2, *P. aeruginosa* escapes recognition by the innate immune systems of both mammals and plants.

Introduction

The innate immune system detects microorganisms and rapidly responds to invasion by eliminating them. Toll-like receptors (TLRs) recognize various evolutionary conserved structures of microorganisms and play a crucial role in innate immune recognition [1]. Stimulation of these receptors triggers intracellular signaling cascades leading to activation of phagocytes and production of pro-inflammatory cytokines. TLRs are type-1 transmembrane proteins characterized by extracellular leucine-rich-repeat motifs and an intracellular Toll/interleukin-1 receptor domain. Dimerization of TLRs is important for activation and ligand recognition, for example TLR2 recognizes diacylated lipopeptides in combination with TLR1 and triacylated lipopeptides together with TLR6. The most studied TLR member is TLR4, which detects the Gram-negative outer membrane component lipopolysaccharide (LPS). TLR5 senses flagellin [2], which is the major component of the bacterial flagellum.

Flagella consist of a basal body, the flagellar hook and a filament which serves as a propeller [3]. The filament consists of 11 protofilaments composed of several thousand flagellin monomers. Flagellin molecules from various bacteria have a conserved N- and C-terminus and a hypervariable central domain. The conserved regions are important in protofilament formation and motility. TLR5 recognizes a conserved part of flagellin that is buried in the flagellar filament and is only accessible in flagellin monomers [4]. By recognizing this part of flagellin, TLR5 detects almost all flagellated bacteria. Mutation of the TLR5-recognition site generally impairs protofilament assembly and thereby motility and virulence [5]. However, in the human pathogens *Campylobacter jejuni* and *Helicobacter pylori* the flagellin is changed in such a way that it is no longer recognized by TLR5, while motility is not affected [6].

Plants have evolved a similar sensing system for flagellin as mammals [7]. In *Arabidopsis thaliana*, stimulation of the plasma membrane-located receptor FLS2 [8] results in the activation of defense responses, such as callose deposition and the production of pathogenesis-related proteins [7]. Flagellin recognition contributes to the resistance of *Arabidopsis* to the bacterial pathogen *Pseudomonas syringae* [9]. Although TLR5 and FLS2 serve a similar function in pathogen recognition, the composition of the receptor, as well as the downstream signaling pathways differ considerably. Furthermore, FLS2 recognizes a different epitope of flagellin than does TLR5. A peptide, called flg22, consisting of 22 amino acids derived from the highly conserved N-terminal region of *P. syringae* flagellin activates FLS2 even better than purified flagellin [7,10].

Pseudomonas aeruginosa is a common environmental Gram-negative bacterium, which acts as an opportunistic pathogen in humans and plants. Normally, the human host counteracts this microorganism effectively via the innate immune system [11]. However, immunocompromised patients, severe burn victims and cystic fibrosis patients are sensitive for *P. aeruginosa* infections. Due to its tendency to colonize surfaces in a biofilm, the bacterium is impervious to therapeutic concentrations of many antibiotics [12].

Detection of *P. aeruginosa* by TLRs activates the innate immune system and protects the host from infection [13]. Flagellin of *P. aeruginosa* is a potent TLR5 activator. It is released during bacterial growth, because the long flagellum tail is easily disrupted [14]. The contribution of TLR5 to the inflammatory response of *P. aeruginosa* may be masked by activation of TLR4 by bacterial LPS [15]. Both receptors cooperate to defend the host from infection: the absence of both TLR4 and TLR5 results in hypersusceptibility for lung infection in mice [16]. Recognition of *P. aeruginosa* flagellin is important for the efficient clearance of the

bacterium in mice [17].
Two extracellular proteases of *P. aeruginosa* that exert their activity at the invasive stage have been associated with virulence i.e. elastase and alkaline protease. Elastase cleaves collagen, IgG, IgA, and some proteins of the complement system [18]. It also degrades fibronectin to expose receptors for bacterial attachment on the mucosa of the lung [19]. Elastase disrupts the respiratory epithelium and interferes with ciliary function. So far, alkaline protease and elastase together are also reported to cause the inactivation of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) [20]. A *Pseudomonas entomophila* strain, that lacks alkaline protease was shown to be less virulent and persistent in a *Drosophila* infection model [21].
Immune evasion is an important strategy for bacteria to survive in their host. Immune evasion molecules are crucial for this survival, as has been demonstrated for several human and plant pathogens [22,23,24,25]. During infection, monomeric flagellin released from bacterial flagella activates TLR5 signaling. To evade innate immune recognition, some bacteria have evolved strategies in which they manipulate flagellin to impair TLR5 activation [6]. We hypothesized that bacteria secrete proteins that interfere with recognition of TLRs. In our search for immune evasion molecules that act as TLRs antagonists, we identified alkaline protease of *P. aeruginosa* as a TLR5 signaling inhibitor.

Materials and Methods

Cell culture and bacterial strains

Dulbecco's modified Eagle's medium (DMEM) and Iscoves modified Dulbecco's medium (IMDM) (Invitrogen), fetal bovine serum (FCS) (Gibco), Human embryonic kidney cells transfected with TLR4/CD14/MD-2 (HEK/TLR4) or TLR5 (HEK/TLR5) and Normocin and Blasticidin (all Invivogen) were used for cell and bacterial culture. Human neutrophils from healthy volunteers were isolated as described [40]. IL-8 ELISA kit and high performance ELISA buffer (HPE) were purchased from Sanquin*. P. aeruginosa* strain PAO25 is a *leu arg* mutant derivative of strain PAO1 [42]. Clinical isolates of *P. aeruginosa*, *S.* Typhimurium, *Staphylococcus haemolyticus*, *Staphylococcus hominis, Staphylococcus warneri, Streptococcus milleri*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Serratia adorigen*, *Listeria monocytogenes* and *Enterobacter cloacae* were obtained within the UMC Utrecht and screened for TLR5 antagonists. Competent *E. coli* TOP10F' and BL21 (DE3) pLys were purchased from Invitrogen and flg22 from Genscript.

HEK/TLR5 assay

HEK/TLR5 cells were maintained in DMEM supplemented with 10% FCS, 10 µg/ml Blasticidin and 100 µg/ml Normocin. Monolayers of HEK/TLR4 and HEK/TLR5 cells were preincubated with 5-fold diluted bacterial supernatant for 30 min and subsequently stimulated with flagellin for 6 h at 37°C. Cell culture supernatant was harvested and stored at -20°C for analysis. Samples were diluted in HPE buffer and IL-8 concentrations were determined by ELISA following manufacturer's protocol, using a standard curve. For some experiments relative IL-8 amounts are expressed as OD at 450 nm.
To measure NF-κB activation, HEK/TLR4 and HEK/TLR5 cells were transiently transfected 2-3 days before stimulation with a NF-κB reporter plasmid pHIV-CAT [43] (AIDS Research and

Reference Reagent Program, Division of AIDS, NIAID, NIH). Transfected cells were stimulated with LPS or flagellin for 5 h at 37°C. Cells were lysed with lysis buffer/substrate (Promega) according to manufacturer's protocol and chemiluminescence was measured using a Centro LB 960 microplate luminometer (Berthold). NF-κB activation is expressed as stimulation index, which represents the ratio between stimulated versus control cells.

Neutrophil assay

Flagellin was incubated with 1 µg/ml AprA in the presence of 10 µg/ml polymyxin B (PMB) (Sigma) for 30 min at 37°C. Neutrophils ($1.25x10^5$/well) were stimulated with AprA-treated flagellin for 16 h at 37°C. Cell culture supernatant was harvested and stored at -20°C for analysis by IL-8 ELISA.

Isolation and purification of AprA

P. aeruginosa (clinical isolate) was cultured overnight in IMDM under constant agitation and supernatant was collected by centrifugation and filtration. Supernatant was applied on a Q sepharose XL column and eluted with PBS + 2 M NaCl pH 7.4 using an Akta FPLC system (GE Healthcare). Active fractions were pooled and concentrated by lyphophilization and resuspended in PBS before gel filtration on a Superdex 75 column (GE Healthcare). Subsequently, active fractions were precipitated with trichloroacetic acid and separated by 12.5% SDS-PAGE gels and stained with silver. Proteins of interest were identified by mass-spectrometry (Alphalyse).

Recombinant AprA was produced in *E. coli* using two plasmids: i) pAG302 (kindly provided by A. de Groot) contains the *aprA* and *aprI* genes under control of the tac promoter on vector pUR6500, a derivative of pMMB67EH containing a kanamycin-resistance cassette; ii) pJF1 (kindly provided by J. Folders) containing the a*prD,* a*prE, and* a*prF* genes under control of the *lac* promoter on vector pBBR1MCS, which contains a chloramphenicol-resistance gene and is necessary for secretion of AprA. The two plasmids were used to transform *E. coli* BL21 and clones resistant to chloramphenicol and kanamycin were selected. Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3-4 h at 37°C. Supernatant was collected and diafiltrated against 20 mM phosphate buffer pH 7 using a proflux M-12 system (Millipore). AprA was applied on a Q sepharose XL column and eluted with 20 mM phosphate-buffered 2 M NaCl pH 7. Fractions containing AprA were concentrated using a 3 kD filter (Millipore) and applied on a Superdex 75 column.

For His-tagged AprA, the gene *aprA* (without the nine residues propeptide) was amplified from genomic PAO1 DNA using a forward and reverse primer with incorporated 5'BamH1 and 3'Not1, respectively. The PCR product was ligated in a modified pET302 vector (Invitrogen) encoding an N-terminal 6x His-tag and transformed in *E. coli* Top10F' cells (Invitrogen). After sequence verification, the constructs were transformed in *E. coli* BL21 and His-tagged proteins was purified under denaturing conditions according to manufacturer's instructions using a His trap column (GE Healthcare). Denatured AprA was diluted 50 times in 0.8 M L-arginine + 50 mM Tris-HCl pH 9 + 1 mM $CaCl_2$ at 4°C overnight [44]. After renaturation, AprA was concentrated on a Amicon 30 kD spin column (Millipore) and washed two times with 50 mM HEPES pH 7.8 + 1 mM $CaCl_2$ + 0.1 mM $ZnCl_2$. Purity was examined with SDS-PAGE and Coomassie staining.

AprI isolation

The gene *aprI* without signal sequence of *P. aeruginosa* strain PAO1 with *Xba*1 restriction site, 6x His-tag and enterokinase cleavage site fused to the 5'-end and a 3' *Eco*R1 restriction site was synthesized by BaseClear. The *aprI* construct was ligated into a pRSETB vector and used to transform *E. coli* Top10F' cells. Protein expression was performed in *E. coli* BL21 and alkaline protease inhibitor was purified under denaturing conditions according to manufacturer's instructions using a His trap FF column (GE Healthcare). Purified denatured protein was diluted 10-fold in PBS and concentrated on a His trap column. Purity of AprI was assessed by SDS-PAGE and the protein was dialyzed against PBS.

Recombinant flagellin isolation

Constructs for recombinant *S.* Typhimurium and *P. aeruginosa* flagellin were generated by an overhang extension polymerase chain reaction (PCR) as described previously [40]. Briefly, genes *fliC* of *S.* Typhimurium (clinical isolate) and flagellin type B of *P. aeruginosa* strain PAO25 were cloned directly downstream of the 6x His-tag and enterokinase cleavage site of the pRSETB vector (Invitrogen). PCRs were performed using VentR DNA polymerase (New England Bio Labs). After verification of the sequence the vector was used to transform *E. coli* BL21 and the protein expression was performed as described for AprA. His-tagged flagellin was isolated by lysing bacteria with cellytic B according to manufacturer's instructions (Sigma) supplemented with DNAse / RNAse and protease inhibitor cocktail (Roche) followed by purification with a His trap FF column. The protein was eluted with 0.5 M imidazole in PBS and dialyzed against PBS. Purity was assessed by SDS-PAGE with Coomassie staining.

Isolation of native flagellar filaments from P. aeruginosa

P. aeruginosa strain PAO25 (flagellin type B) and a clinical isolate (flagellin type A) were grown overnight in Luria-Bertani broth and bacteria were pelleted by centrifugation. Pellets were resuspended in PBS and flagella were sheared from bacteria by blending, followed by centrifugation at 8,000 g for 15 min to pellet the bacteria. Flagella were collected from the supernatant by centrifugation at 100,000 g for 60 min. The pellets obtained were resuspended in PBS and purity was examined by SDS-PAGE. Isolated flagella were heated at 70°C for 20 min for depolymerization.

P. aeruginosa transposon mutants

P. aeruginosa mutants *aprA1* (3969) and *aprA2* (16254) were obtained from the *P. aeruginosa* PAO1 transposon mutant library [45] (University of Washington). Transposon insertions in the *aprA* mutant strains were confirmed by PCR. Bacteria were grown overnight in IMDM and supernatant was harvested by centrifugation and filtration. Where indicated, 3 µg/ml recombinant AprA or 10 µg/ml AprI was added to the culture medium before inoculation with the strains. HEK/TLR5 cells were stimulated with dilutions of these overnight culture supernatants and IL-8 release was measured by ELISA. For neutrophil stimulation bacterial supernatant was incubated with 10 µg/ml PMB for 30 min at 37°C. Neutrophils were stimulated with untreated and PMB-treated bacterial supernatant for 16 h at 37°C. Cell culture supernatant was harvested and IL-8 concentration was determined by ELISA.

Flagellin and flg22 degradation

Recombinant AprA, AprI and flagellin of *P. aeruginosa* or *S.* Typhimurium in PBS were incubated for 1 h (unless specified otherwise) at 37°C. Cleavage products were analyzed by SDS-PAGE and stained with Coomassie. AprA was inhibited by preincubation with 100 mM EDTA or AprI before addition of flagellin. Flg22 was incubated with 1 µg/ml AprA for 1 h at 37°C. The sample was spotted on a NP-20 array (Biorad) and analyzed using a ProteinChip SELDI reader (Biorad).

AprA detection

The presence of AprA in supernatants of *P. aeruginosa* mutants was checked by Western blotting using a polyclonal rabbit AprA antiserum (generously provided by R. Voulhoux). Recombinant AprA or bacterial supernatant was separated with SDS-PAGE. Proteins were transferred to an Immobilon-P membrane (Millipore) and blocked with 4% skimmed milk in PBS + 0.05% Tween. Subsequently blots were incubated with 1/500 diluted rabbit AprA antiserum followed by HRP-conjugated goat-anti-rabbit IgG (Biorad) and bands were visualized by enhanced chemiluminescence (Amersham).

Arabidopsis callose deposition assay

Seeds of *A. thaliana* ecotype Landsberg *erecta* (La-*er*) were vapor face sterilized and sown on Murashige-Skoog (MS; Sigma) medium containing 0.6% Plant Agar (Duchefa) and 1% (w/v) sucrose (Sigma). After a two-day vernalization period at 4°C, plates were transferred to growth chambers with an 8 h day (200 $\mu Em^{-2}.sec^{-1}$ at 24°C) and 16 h night (20°C) cycle for seven days. Three seedlings were transferred to a single well containing 1 ml MS containing 1% (w/v) sucrose and the components required for the treatments as indicated in the text. After 24 h the medium was replaced by 1 ml 96% EtOH followed by incubation overnight for removal of chlorophyll. The next day, decolorized seedlings were washed in 0.07 M phosphate buffer (pH 9) and subsequently incubated with the same buffer containing 0.01% aniline blue (water blue; Merck). Samples were placed in the dark for a period of 20 h at RT. Microscopic slides were prepared in a matrix of fresh aniline blue. Observations were performed with a fluorescence microscope (Olympus Ax70 with Olympus U-RFL-T) with UV filter (bandpass 340 to 380 nm, long-path 425 nm) [46].

Arabidopsis growth assay

Ten vapor-face sterilized seeds of *A. thaliana* (La-*er*) were transferred to a well of 24-well plates containing 1 ml MS medium with 1% (w/v) sucrose and treatment-specific components. Seeds were vernalized by putting the 24-well plates at 4°C for two days. Subsequently, plates were transferred to growth chambers with an 8-h day (200 $\mu Em^{-2}.sec^{-1}$ at 24°C) and 16 h night (20°C) cycle. Differences in growth rate were monitored after 7-10 days by photography.

Stomatal closure assay

Wild-type *A. thaliana* Col-0 plants were grown for 5 weeks in an autoclaved mixture of sand/potting soil in a growth chamber with a 9 h day (200 $\mu E\ m^{-2}\ s^{-1}$ and 24°C) and 15 h night (20°C) cycle and 70% relative humidity as described [47]. Wild-type *P. aeruginosa* PAO1 *aprA* mutant strains were grown overnight in liquid Kings Medium B at 28°C. The leaves of

5-week-old plants were dipped for 2 seconds in a solution of 10 mM $MgSO_4$ and 0.015% (v/v) Silwet containing $2.5 \cdot 10^7$ cfu/ml of *P. aeruginosa* bacteria. The epidermis of two leaves were peeled off before treatment (t=0) and 5, 10, 20 and 40 min after treatment and immediately observed under a Zeiss Axioskop2 microscope (400x). Pictures were taken at 10-15 random regions of the leaves. Stomatal aperture was then measured using the software package ImageJ.

Accession numbers

Swiss-prot accession numbers: *P. aeruginosa* AprA (Q03023), AprI (Q03026), flagellin type B (P72151) and flagellin from *S.* Typhimurium (P06179).

Results

A secreted protein of P. aeruginosa inhibits TLR5 activation

To identify TLR5 inhibitors, we screened several culture supernatants of Gram-positive and Gram-negative bacteria for inhibitory activity on activation of TLR5-transfected HEK cells (HEK/TLR5) containing an NF-κB luciferase reporter. Stimulation of HEK/TLR5 with flagellin triggers the activation of NF-κB and the secretion of Interleukin 8 (IL-8) into culture supernatants. These cells are insensitive for the TLR4 ligand LPS, whereas they respond to monomeric flagellin in the picomolar range (Fig. 1A and 1B). Most bacterial supernatants did not significantly inhibit IL-8 production upon flagellin stimulation. However, the supernatant of *P. aeruginosa* reduced activation of HEK/TLR5 cells consistently with about 30%. Therefore, this supernatant was chosen to isolate the potential TLR5 inhibitor.

To purify the inhibitory compound from *P. aeruginosa*, we fractionated the supernatant with ion-exchange chromatography. In an agonist dose response experiment, specifically eluted fractions inhibited IL-8 production completely when the HEK/TLR5 cells were stimulated

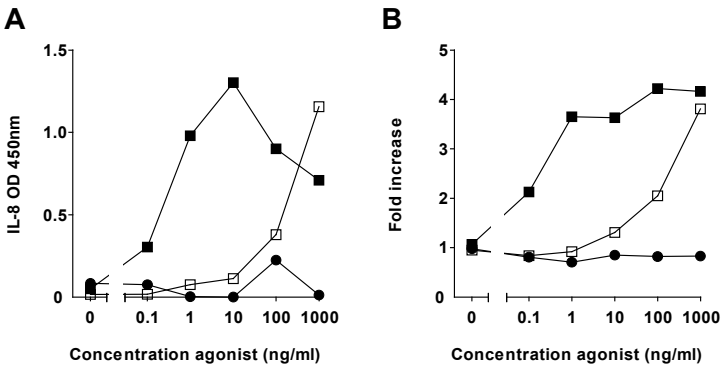

Figure 1. Fractionated *P. aeruginosa* supernatant inhibits TLR5 activation

HEK/TLR5 cells were transfected with a NF-κB reporter construct. Cells were incubated with 20-fold diluted elution fraction from a Q sepharose column (inhibitor) for 30 min and subsequently challenged with various concentrations recombinant flagellin of *S.* Typhimurium (□). Flagellin without inhibitor (■) and LPS (●). (A) After 6 h the IL-8 concentration in the cell culture supernatant was measured by ELISA. (B) NF-κB activation was determined by measuring luciferase activity in a luminometer and expressed as fold increase of luciferase activity over stimulation with culture medium alone. The presented data are representative for the inhibition of TLR5 signaling that is typically observed with purifications of *P. aeruginosa* supernatant.

with up to 10 ng/ml flagellin of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (Fig. 1A). In a parallel independent assay, the same fractions inhibited flagellin-stimulated NF-κB activation (Fig. 1B). LPS-stimulation of HEK/TLR4 cells was not affected by the partially purified *P. aeruginosa* supernatant (Fig. S1), which suggests that the inhibitor acts at the receptor level and not at downstream signaling routes. Additional purification of the inhibitory activity by size-exclusion chromatography resulted in one band by SDS-PAGE that correlated with TLR5-inhibiting activity of eluted fractions (Fig. S2). We identified, using mass-spectrometry, the protein of interest as alkaline protease.

AprA inhibits TLR5 activation

The gene *aprA* of *P. aeruginosa* encodes alkaline protease (designated AprA), which is a 50 kD zinc metalloprotease [26]. AprA is secreted via a type I secretion system, which is encoded by the genes a*prD*, *aprE* and *aprF* [27] (Fig. 2A). The gene downstream of *aprA*, a*prI*, encodes a highly specific inhibitor of AprA [28]. Some biological substrates of AprA have been described, such as the cytokine IFN-γ [20]. To verify that AprA inhibits TLR5-mediated cell activation, we cloned and expressed *aprA* with a 6x His-tag or together with the genes encoding the secretion apparatus in *Escherichia coli* and purified AprA from the medium. The TLR5 inhibitory activity of recombinant His-AprA and secreted recombinant AprA (data not shown) was comparable to that of purified AprA from *P. aeruginosa* (Fig. 1A and 2B). Incubation of HEK/TLR5 cells with recombinant AprA abolished IL-8 production completely even when the cells were stimulated with flagellin concentrations up to 100 ng/ml (Fig. 2B). To determine the potency of AprA, different concentrations were tested for inhibition of the flagellin-induced IL-8 production by HEK/TLR5 cells. Complete inhibition of cell activation by 30 ng/ml *P. aeruginosa* flagellin was observed with 0.3 μg/ml AprA with a half maximal inhibitory concentration of 30 ng/ml (Fig. 2C). Flagellin isolated from *P. aeruginosa* also served as a potent stimulator of HEK/TLR5 cells. As for *S.* Typhimurium flagellin, AprA completely inhibited the *P. aeruginosa* flagellin response (Fig. 2D). HEK/TLR5 cells are unresponsive to lipopolysaccharide, a contaminant of recombinant proteins isolated from *E. coli*, in contrast to naturally TLR5 sufficient cells like human neutrophils. To investigate the effect of AprA on TLR5 activation of human neutrophils, we incubated *P. aeruginosa* flagellin with AprA in the presence of polymyxin B, which neutralizes LPS activity. In addition to HEK/TLR5 cells, treatment of *P. aeruginosa* flagellin with 1 μg/ml AprA inhibited the IL-8 production of neutrophils completely (Fig. 2E). These data demonstrate that AprA from *P. aeruginosa* is an inhibitor of TLR5-mediated cell activation by different types of flagellin.

AprA cleaves flagellin

To determine the mechanism of TLR5 inhibition, we investigated the effect of AprA on TLR5 and flagellin separately. To study any direct effect of AprA on TLR5, cells were incubated with the protease for 30 min, washed and subsequently stimulated with flagellin. A washing step between AprA incubation and addition of flagellin preserved TLR5 activation (Fig. 3A). This result indicates that TLR5 is not affected by AprA and still properly responds to flagellin. Another possible mechanism to interfere with TLR5 recognition is neutralization or proteolysis of flagellin. To test this hypothesis, isolated flagellin was incubated with AprA and degradation was analyzed by SDS-PAGE. Flagella from *P. aeruginosa* are composed of either type A or B flagellin, depending on the strain. These two flagellins contain a

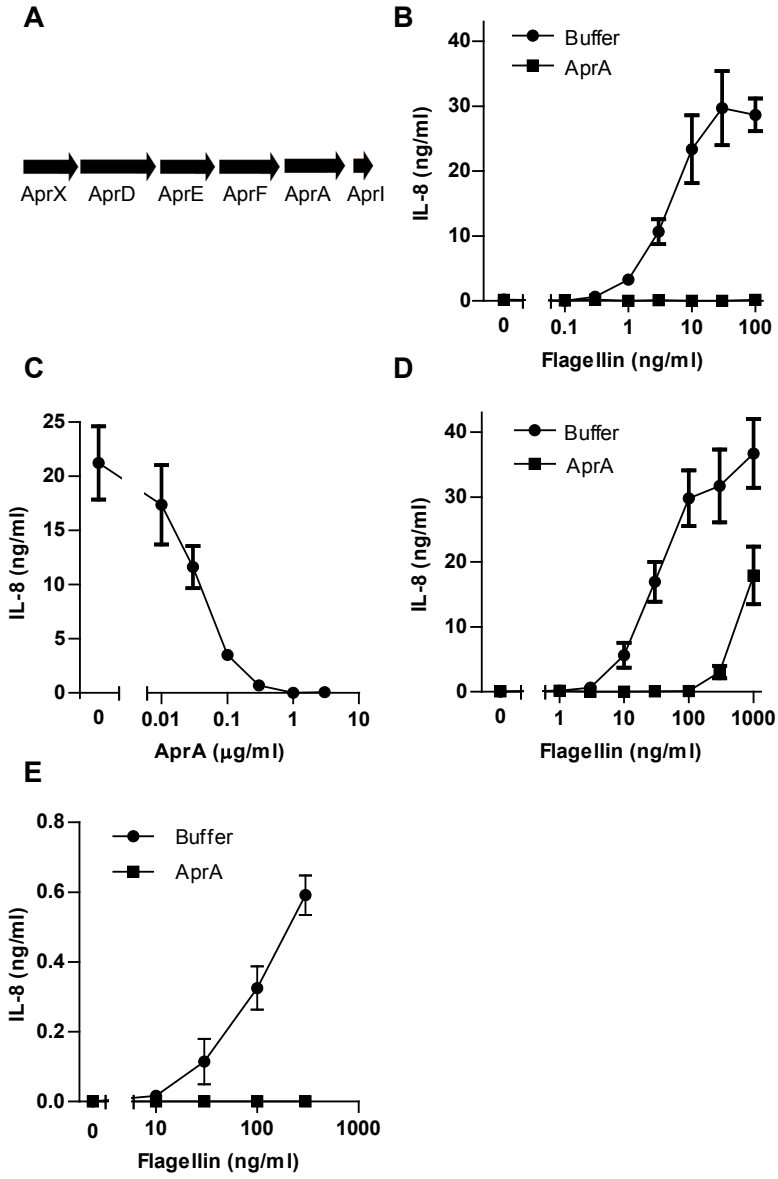

Figure 2. AprA prevents flagellin-induced IL-8 production by HEK/TLR5 cells
(A) Schematic representation of the gene cluster of *aprA*, *aprI*, genes involved in secretion, *aprD*, *aprE*, and *aprF* and *aprX* on the genome of *P. aeruginosa* strain PAO1. (B) Different concentrations of recombinant flagellin from *S.* Typhimurium were treated with 1 µg/ml recombinant His-AprA for 30 min and subsequently added to HEK/TLR5 cells. After 6 h IL-8 was measured in the supernatant by ELISA. (C) His-AprA concentration-dependent inhibition of flagellin-induced HEK/TLR5 cell activation. HEK/TLR5 cells were treated with varying concentrations of AprA and challenged with 30 ng/ml flagellin of *P. aeruginosa.* (D) Recombinant flagellin of *P. aeruginosa* was incubated with buffer or 1 µg/ml His-AprA for 30 min and subsequently added to HEK/TLR5 cells for IL-8 release. (E) Recombinant flagellin of *P. aeruginosa* was incubated with 1 µg/ml recombinant His-AprA in the presence of PMB (10 µg/ml) for 30 min at 37°C, and subsequently added to human neutrophils. After 16 h IL-8 concentration was measured by ELISA. Results represent mean IL-8 concentration ± SEM from three independent experiments.

completely different variable domain, but showed comparable degradation patterns upon incubation with increasing concentrations AprA (Fig. 3B and 3C). An identical cleavage pattern was observed for recombinant *P. aeruginosa* flagellin type B (Fig. 3D). Moreover, AprA cleaved flagellin from another species i.e. *S.* Typhimurium (Fig. 3E). Degradation of flagellin was time-dependent and started within one minute after addition of AprA (Fig. 3F). Cleavage of flagellin occurred in multiple steps, dependent on the protease concentration and incubation time. At higher concentrations of AprA, flagellin type A (Fig. 3B) and *S.* Typhimurium (Fig. 3E) flagellin were completely degraded, while for *P. aeruginosa* flagellin type B (Fig. 3C and 3D) a truncated protein of about 37 kD remained visible. The proteolytic activity of AprA is inhibited by 100 mM EDTA [29]. In our experiments EDTA also abolished

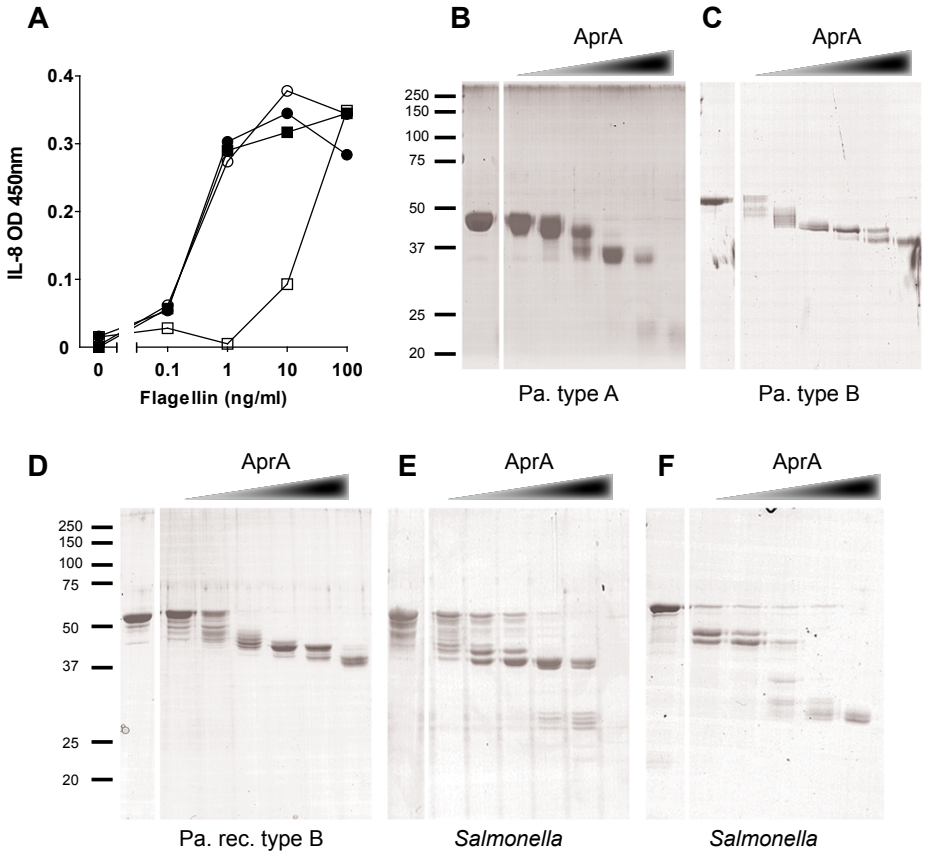

Figure 3. AprA of *P. aeruginosa* cleaves flagellin
(A) HEK/TLR5 cells were treated for 30 min with AprA purified from *P. aeruginosa* culture supernatant and directly stimulated (□) or washed (○) before stimulation with varying concentrations flagellin from *S.* Typhimurium. As control cells were directly stimulated (●) or washed (■) before addition of flagellin. After 6 h incubation, IL-8 was measured in the supernatant of HEK/TLR5 cells by ELISA. (B-F) Degradation of flagellin by recombinant AprA. Flagellin was mixed with 0, 0.01, 0.03, 0.1, 0.3, 1 and 3 μg/ml AprA for 60 min at 37°C in PBS and protein degradation was analyzed by SDS-PAGE and Coomassie staining. Cleavage of flagellin by AprA was compared for native monomeric (B) flagellin type B isolated from *P. aeruginosa* strain PAO25 (1 mg/ml), (C) flagellin type A isolated from clinical *P. aeruginosa* strain (150 μg/ml), (D) recombinant flagellin type B from *P. aeruginosa* (250 μg/ml) and (E) recombinant flagellin of *S.* Typhimurium (250 μg/ml). (F) Time-dependent degradation of flagellin by AprA. Flagellin (250 μg/ml) of *S.* Typhimurium was incubated with AprA for 0, 1, 3, 10, 30 and 60 min at 37°C in PBS.

degradation of flagellin (Fig. S3). The optimum pH for AprA is pH 9-10 [18]; however in our experiments flagellin was efficiently degraded under physiological conditions. These findings demonstrate that *P. aeruginosa* secretes a protease that efficiently degrades flagellin of different species and thereby prevents activation via TLR5.

Flagellar filaments are not degraded by AprA

Flagellin is the most abundant protein of the flagellum, which is essential for bacterial motility and virulence. Secretion of a protease by *P. aeruginosa* that degrades the flagellum would be disadvantageous for the bacterium. Therefore, we investigated whether AprA also degrades complete flagellar filaments isolated from *P. aeruginosa*. These filaments consist of polymerized flagellin. Incubation of flagellin polymers with AprA did not result in degradation of flagellin (Fig. 4A). However, after depolymerization, the resulting monomeric flagellin was degraded by AprA. Even at higher concentrations AprA did not cleave flagellar filaments as observed for monomeric flagellin. This indicates that AprA inactivates only monomeric flagellin in the surrounding of the bacterium, while the integrity of flagella is preserved.

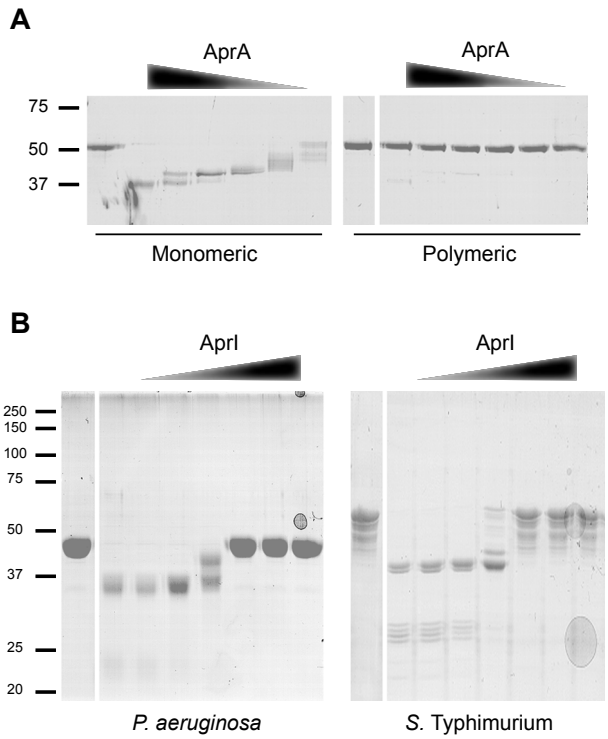

Figure 4. Polymeric flagellin resists cleavage by AprA and AprI is an efficient endogenous inhibitor
(A) Flagella isolated from PAO1 were treated for 20 min at 70°C to obtain monomeric flagellin. Untreated polymeric flagellin was compared with monomeric flagellin for susceptibility to AprA cleavage. Monomeric and polymeric flagellin was incubated with 0, 3, 1, 0.3, 0.1, 0.03 or 0.01 µg/ml AprA for 60 min at 37°C and analyzed by SDS-PAGE. (B and C) AprA (1 µg/ml) was incubated with 0, 0.03, 0.1, 0.3, 1, 3 or 10 µg/ml AprI and subsequently flagellin of (B) *P. aeruginosa* or (C) *S.* Typhimurium was added. Samples were analyzed by SDS-PAGE, untreated flagellin control is shown in the first lane followed by increasing AprI concentrations.

AprI blocks AprA-mediated cleavage

Downstream of the *aprA* gene, *aprI* is located which, encodes for alkaline protease inhibitor (designated AprI). Kinetic studies revealed a very high affinity of this 11.5 kD protein for AprA [28]. To investigate whether AprI interferes with AprA-mediated cleavage, we cloned and expressed the *aprI* gene in *E. coli* as a His-tagged protein and purified it using Ni-affinity chromatography. AprI prevented cleavage of flagellin slightly at equimolar concentrations (Fig. 4B and C). Flagellin cleavage was completely blocked at higher AprI concentrations. AprI protected both *P. aeruginosa* and *S.* Typhimurium flagellin against AprA-mediated proteolysis. Importantly, incubation of AprA with AprI before addition of flagellin restored activation of HEK/TLR5 cells dose-dependently (Fig. S4). In conclusion, AprI of *P. aeruginosa* is a very potent inhibitor of AprA-mediated cleavage of flagellin.

Pseudomonas aprA mutant activates TLR5

As demonstrated above, purified recombinant AprA effectively blocked flagellin-induced TLR5 activation. To address the importance of this protease in a more natural environment, we compared the TLR5-activating capacity of culture supernatants from *P. aeruginosa* wild-type (WT) and *aprA* transposon-insertion mutants. In this setup, we tried to understand the dynamic interaction between the endogenous flagellin and AprA when released simultaneously by growing bacteria. Only the supernatant of the *aprA* mutant strains triggered TLR5 signaling (Fig. 5A). Strikingly the supernatant of the wild-type strain did not initiate IL-8 production at all. This experiment indicates that AprA completely degraded flagellin monomers that were released in the supernatant during overnight growth. As expected, dilution of supernatants of the *aprA* mutants limited TLR5 activation due to a decrease in flagellin concentration (Fig. 5A). To show that the absence of AprA is responsible for the TLR5-activating capacity in the *aprA* mutant strains, we supplemented the culture medium of these strains with recombinant AprA before inoculation. This resulted in the same lack of activation as for the wild-type strain supernatant (Fig. 5B), demonstrating that AprA is essential as well as sufficient for degradation of flagellin. Complementary, addition of AprI to the culture medium abolished AprA-mediated cleavage of flagellin in the wild-type strain resulting in activation of TLR5 to comparable levels of that of the *aprA* mutant strains (Fig. 5C). These results show that the wild-type strain does release flagellin in its environment. To investigate the inflammatory response of naturally TLR4 and TLR5 sufficient cells, we stimulated neutrophils with bacterial supernatant of wild-type and an *aprA* mutant strain. As for HEK-TLR5 cells, the aprA mutant strain triggered higher IL-8 production in comparison to wild-type *P. aeruginosa* (Fig. 5D). Addition of polymyxin B, to neutralize LPS, slightly inhibited IL-8 production of neutrophils in response to supernatant of wild-type and the *aprA* mutant strain, without changing the difference in IL-8 production between the two strains. To verify that the *aprA* mutant strains did not produce AprA, the culture supernatant was probed with a specific antiserum against AprA by western blotting. As expected, the wild-type strain secreted AprA while the two *aprA* mutant strains did not (Fig. 5E). Growth and motility of the mutant strains was comparable with wild-type. Together, these results demonstrate that *P. aeruginosa* secretes sufficient AprA to neutralize its own monomeric flagellin for detection via TLR5.

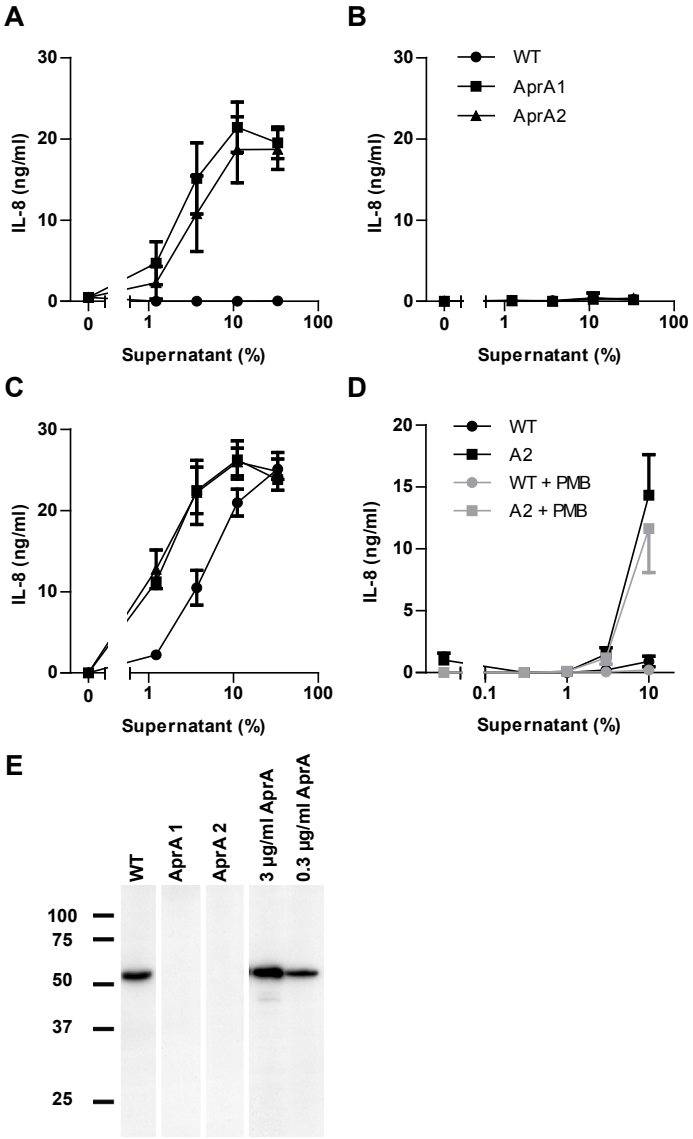

Figure 5. Culture supernatant of *aprA* mutant strains trigger TLR5

(A) Dilutions of bacterial culture supernatants, collected from overnight grown wild-type (WT), and isogenic *aprA* mutant strains were used as flagellin source to stimulate HEK/TLR5 cells for IL-8 production. (B) 3 µg/ml recombinant AprA was added to the culture medium before inoculation with WT, *aprA*1 or *aprA*2 mutant strains. HEK/TLR5 cells were incubated with dilutions of bacterial culture supernatants and stimulated for 6 h. IL-8 production was measured by ELISA. All three data sets completely overlap in this graph. (C) Wild-type and mutant *P. aeruginosa* strains were grown in the presence of 10 µg/ml exogenous AprI and dilutions of the culture supernatants were added to HEK/TLR5 cells for IL-8 release. Data are expressed as mean IL-8 concentration ± SD from triplicates. (D) Human neutrophils were stimulated with dilutions of bacterial culture supernatants of wild-type and *aprA*2 mutant strain. PMB (10 µg/ml) was added prior to stimulation for 30 min at 37°C. After 16 h stimulation, IL-8 concentration in cell supernatant was determined. Results represent mean ± SEM of three independent experiments. (E) Culture supernatant of overnight grown wild-type and *aprA* mutant strains or recombinant AprA were analyzed for the presence of AprA by immunoblotting.

AprA interferes with flagellin recognition by Arabidopsis

Like mammals, plants also possess an innate immune system in which the detection of flagellin monomers results in the activation of effective immune responses [30]. In *Arabidopsis*, the FLS2 receptor has been demonstrated to specifically interact with a conserved 22-amino acid motif (flg22) of flagellin monomers [31]. Upon interaction of FLS2 with flagellin monomers or flg22, several downstream defense mechanisms are activated, amongst which the deposition of callose polymers [10]. Furthermore, as a result of the activation of energy-costly defense mechanisms, treatment with flagellin or flg22 has a negative effect on *Arabidopsis* growth [10]. To assess whether AprA activity can also prevent flagellin-induced defense activation in plants, we studied the effect of AprA on flagellin- or flg22-induced callose deposition and growth inhibition of *Arabidopsis* seedlings. *P. aeruginosa* flagellin monomers triggered callose deposition (Fig. 6A) and affected growth (Fig. S6) to a slightly less extent than did flg22, which is in line with earlier observations [30]. Preincubation of flagellin or flg22 with AprA abolished callose deposition (Fig. 6A) and restored plant growth to control levels (Fig. S5). This result indicates that AprA disrupts the active epitope of flagellin that is normally recognized by FLS2. Moreover, AprA completely neutralized the effects of the peptide flg22, these results suggest that AprA cleaves flagellin at least within this 22-amino acids long conserved motif. Therefore, we examined the mass of protease-treated flg22 and indeed observed clear degradation of the peptide by SELDI-TOF (Fig. S6). Addition of AprI prior to AprA treatment of flagellin neutralized the AprA-mediated effects in *Arabidopsis*, while AprI had no effect when added after AprA treatment of flagellin (Fig. 6A and S5).

AprA delays early plant immune responses

The epidermis of plant leaves contains many pores (stomata) of which the aperture is dependent on environmental factors such as humidity and CO_2 concentration [32]. Previously, Melotto et al. [33] showed that besides these environmental cues, recognition of bacterial PAMPs, such as flagellin and LPS, trigger plant immune responses that lead to rapid stomatal closure (Fig. 6B). In this way, the stomata have an important early defense function that actively prevents bacteria from entering the host [33]. We hypothesized that degradation of flagellin by AprA likely affects the speed of stomatal closure upon bacterial inoculation. Therefore, we monitored the stomatal aperture of *A. thaliana* leaves after inoculation with wild-type and mutant *P. aeruginosa* bacteria. Fig. 6C shows that both wild-type PAO1 and the *aprA* mutant strain triggered closure of the stomata within 40 minutes. However, the stomatal aperture decreased significantly faster after treatment with the *aprA* mutant strain. Hence, we conclude that AprA produced by wild-type bacteria plays an important role in the evasion of host immunity of *A. thaliana* by hampering closure of the natural pores that are crucial for bacterial invasion.

Discussion

P. aeruginosa produces various proteases that degrade several host proteins and are associated with virulence [20]. Alkaline protease of *P. aeruginosa* is involved in suppression of the immune response by degradation of cytokines including TNF-α and IFN-γ. Screening for bacterial TLR5 inhibitors resulted in the identification of AprA of *P. aeruginosa*. In this

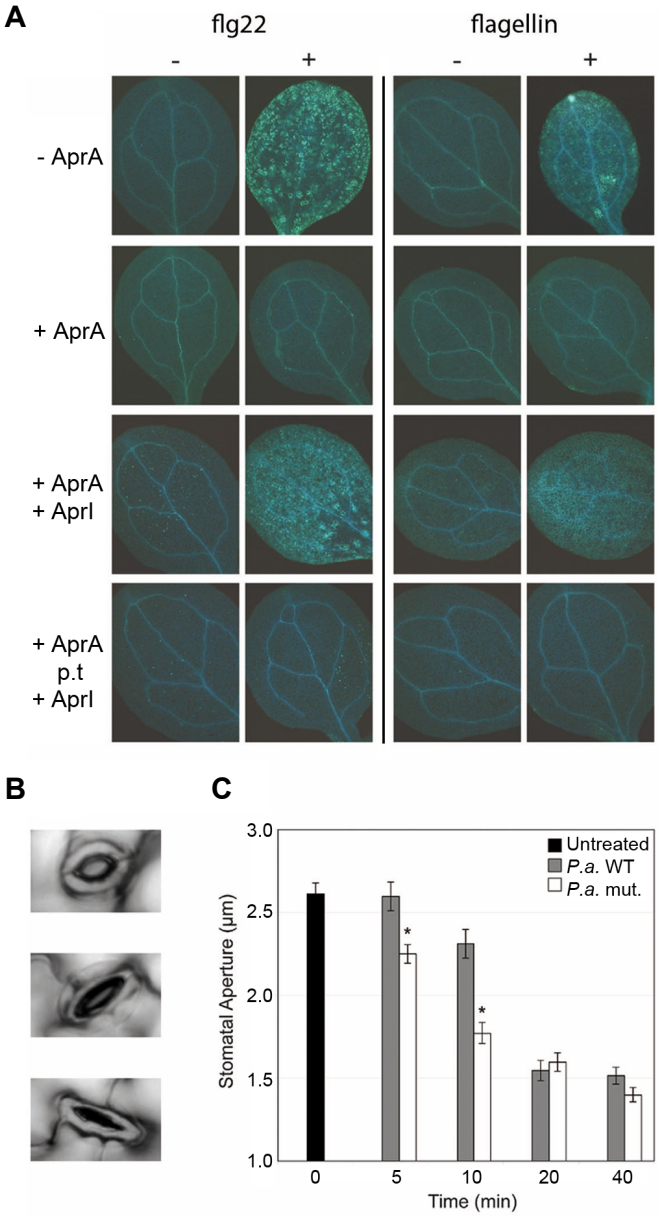

Figure 6. AprA prevents recognition of flagellin in *Arabidopsis* and prevents stomatal closure
A. thaliana La-*er* seedlings were incubated with or without 500 nM flg22 or *P. aeruginosa* flagellin preincubated with 3 µg/ml AprA when indicated. (A). After treatment for 24 h, seedlings were stained for callose deposition by aniline blue and fluorescence was photographed under UV light. In the 3rd row panels AprI was added before AprA treatment and in the bottom panels post AprA treatment (p.t.) of flagellin. (B) Examples of open (top), half open (middle) and closed (bottom) stomata, that were observed during the experiment. (C) Stomatal aperture on leaves of 5-week-old *A. thaliana* plants up to 40 minutes after treatment with *P. aeruginosa* PAO1 or isogenic *aprA* mutant strain (n=108 to 224). Error bars indicate SEM. Asterisks indicate significant differences (Student's t-test; $p<0.001$) between WT and AprA mutant treated plants.

study, we describe flagellin as an AprA substrate. TLR5 is unaffected by the proteolytic activity of this metalloprotease, however its ligand flagellin is degraded effectively and loses stimulatory activity

The only known ligand for TLR5 is bacterial flagellin, which triggers the production of pro-inflammatory cytokines. Only monomeric flagellin activates TLR5 signaling, while in the polymeric form, which is present in flagella, the TLR5 recognition site is inaccessible. Previous studies showed that the N- and C-terminus of flagellin are located inside the flagellar filament [34]. This highly conserved part of flagellin activates TLR5 and is essential for filament assembly. Here we show that AprA fails to degrade polymeric flagellin and therefore does not affect motility. In this way, *P. aeruginosa* protects its functional flagellin present in flagella, while it neutralizes TLR5-activating monomeric flagellin (Fig. 7). The proteolytic activity of AprA can be inhibited with EDTA [29] and with a natural inhibitor of *P. aeruginosa*, AprI, which blocks the catalytic site of AprA [26]. We demonstrate that AprI prevents AprA-mediated flagellin cleavage. The physiological role for AprI is unclear. An obvious possibility is that it protects intracellular bacterial proteins from degradation.

Bacterial release of monomeric flagellin [14] results in the activation of TLR5. Since picomolar concentrations of flagellin can trigger TLR5, highly efficient degradation is a prerequisite to abolish TLR5 signaling. *P. aeruginosa* produces sufficient amounts of AprA to degrade its own released flagellin completely, resulting in avoidance of TLR5 activation. Mutant *aprA* strains of *P. aeruginosa* lacking AprA illustrate that AprA is responsible for this effect, because supernatants of these strains activate TLR5 signaling. This is best demonstrated in our experiments where we use supernatants of wild-type bacteria that contain endogenous amounts of flagellin and AprA (Fig. 5A). No TLR5 activation was

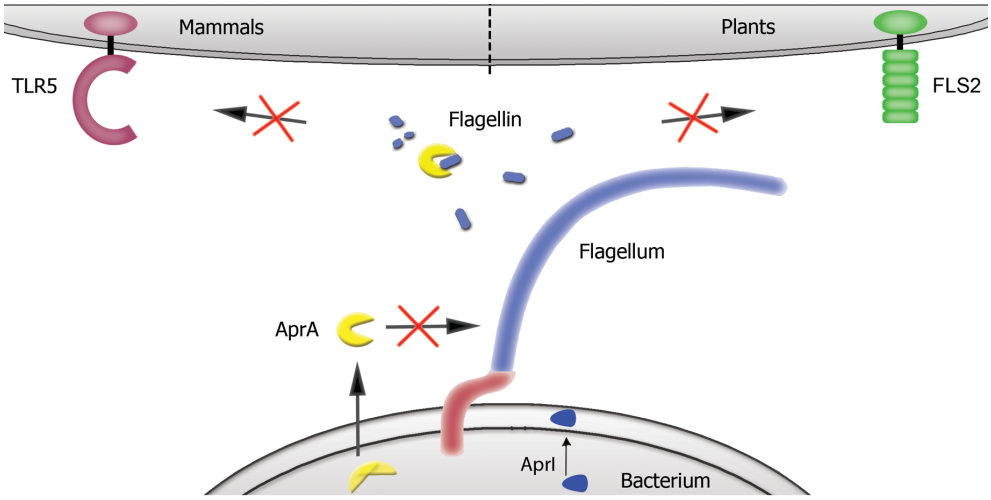

Figure 7. Proposed mechanism for AprA

P. aeruginosa secretes AprA, which degrades free monomeric flagellin in the surrounding of the bacterium, whereas polymeric flagellin present in flagella is not affected. In this way flagellin is not recognized by TLR5 and FLS2, and thereby *P. aeruginosa* escapes activation of the innate immune system in both mammals and plants. AprA is secreted in one step over both membranes and is not present in its active form in the cytoplasm of the bacterium.

observed in these experiments, while the same experiments with mutant strains lacking AprA showed high flagellin-mediated stimulatory capacity. The effect of TLR4 and TLR5 in the host defense against *P. aeruginosa* was shown to be redundant [13,16]. However, TLR5 is the only TLR that is significantly higher expressed on neutrophils in the cystic fibrosis lung [35]. In addition, flagellin triggers phagocytosis of *P. aeruginosa* and oxidative burst by peripheral blood neutrophils. Supernatant of an *aprA* mutant enhanced the inflammatory response of human neutrophils in comparison to wild-type *P. aeruginosa* supernatant. This suggests that flagellin-mediated TLR5 activation is important to stimulate an inflammatory response against *P. aeruginosa*, which is inhibited by AprA. Liehl et al. [21] recently reported that an *aprA* knockout strain of *Pseudomonas entomophila* showed decreased virulence in a *Drosophila* infection model. In their model, *P. entomophila* AprA is necessary to persist in the host and for pathogenicity. Moreover, AprA protected against the *Drosophila* immune response. Although no homolog of TLR5 is described in *Drosophila*, flagellin does trigger the production of antimicrobial peptides [36]. It is tempting to speculate that the immune evasion strategy of *P. aeruginosa* is also operational in this model. In this scenario, escape of innate immune detection by AprA-mediated degradation of pathogen-derived monomeric flagellin in the environment, avoids the production of antimicrobial peptides by the host, which results in a more persistent infection with *P. entomophila*.

In plants we observe the same phenomenon. The FLS2 flagellin receptor in *Arabidopsis* recognizes a different epitope of flagellin then does TLR5. By affecting the ligand instead of targeting the receptor, *P. aeruginosa* evades recognition of flagellin by both FLS2 and TLR5. The same was true for the flagellin-derived peptide flg22, which stimulates FLS2. Stomatal closure is important to prevent bacteria from entering the plant and recognition of flagellin by FLS2 plays a profound role in this response [37]. *P. aeruginosa* lacking a functional *aprA* gene triggers faster closure of stomata in comparison with wild-type. Thus, AprA interferes with this early defense response. Although *P. aeruginosa* itself is not a true plant pathogen, other *Pseudomonas* species that infect *Arabidopsis* like *P. syringae* also contain an *apr* operon. AprA and its inhibitor AprI of *P. syringae* share similarity with AprA and AprI of *P. aeruginosa* of 71% and 50%, respectively. Hence, evasion of TLR5 and FLS2 recognition as we observed for *P. aeruginosa* may be a broad mechanism utilized by various bacterial species.

AprA belongs to the superfamily of metzincin metalloproteases and to the family of serralysins [38]. Proteases from *Serratia marcescens* and *Erwinia chrysanthemi* belong to the family of serralysins and share high sequence similarity with AprA [39]. These bacteria also possess a highly specific protease inhibitor and a similar secretion system for AprA. In these flagellated bacteria AprA homologs may degrade flagellin in the same way as observed for *P. aeruginosa*. AprA cleaves flagellin from both *P. aeruginosa* and *S.* Typhimurium. Since the cleavage site of AprA is within the conserved domain of flagellin, cleavage of flagellin from other flagellated bacteria can be expected.

Innate immune defense systems recognize evolutionary conserved structures. Evasion of immune receptors is a smart strategy of pathogens to escape activation of the host innate immune response [40,41]. For a bacterium with a broad host range it is of great advantage to evade activation of the innate immune system by degrading the ligand that is recognized by the host. Here we provide evidence that *P. aeruginosa* has evolved such a system to circumvent TLR5 and FLS2 activation by flagellin degradation. The consequence is that the benefit for the bacterium, in this case intact flagella and movement should be protected. By

secreting AprA, which is specific for monomeric form of flagellin, *Pseudomonas* has tackled this, without affecting the structure of flagellin in the flagellum. In this way *Pseudomonas* can evade the activation of pattern-recognition receptors such as TLR5 and FLS2 signaling, creating a window of opportunity to evade the immune system of different hosts and cause disease.

Acknowledgements

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References

1. Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783-801.
2. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, et al. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410: 1099-1103.
3. Chevance FF, Hughes KT (2008) Coordinating assembly of a bacterial macromolecular machine. Nat Rev Microbiol 6: 455-465.
4. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, et al. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 4: 1247-1253.
5. Andersen-Nissen E, Smith KD, Bonneau R, Strong RK, Aderem A (2007) A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin. J Exp Med 204: 393-403.
6. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, et al. (2005) Evasion of Toll-like receptor 5 by flagellated bacteria. Proc Natl Acad Sci U S A 102: 9247-9252.
7. Gomez-Gomez L, Boller T (2002) Flagellin perception: a paradigm for innate immunity. Trends Plant Sci 7: 251-256.
8. Robatzek S, Chinchilla D, Boller T (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. Genes Dev 20: 537-542.
9. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, et al. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428: 764-767.
10. Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. Plant J 18: 277-284.
11. Lyczak JB, Cannon CL, Pier GB (2000) Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. Microbes Infect 2: 1051-1060.
12. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318-1322.
13. Skerrett SJ, Liggitt HD, Hajjar AM, Wilson CB (2004) Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against Pseudomonas aeruginosa but not Staphylococcus aureus. J Immunol 172: 3377-3381.
14. Nouwens AS, Willcox MD, Walsh BJ, Cordwell SJ (2002) Proteomic comparison of membrane and extracellular proteins from invasive (PAO1) and cytotoxic (6206) strains of Pseudomonas aeruginosa. Proteomics 2: 1325-1346.
15. Feuillet V, Medjane S, Mondor I, Demaria O, Pagni PP, et al. (2006) Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. ProcNatlAcadSciUSA 103: 12487-12492.
16. Ramphal R, Balloy V, Jyot J, Verma A, Si-Tahar M, et al. (2008) Control of Pseudomonas aeruginosa in the lung requires the recognition of either lipopolysaccharide or flagellin. J Immunol 181: 586-592.
17. Balloy V, Verma A, Kuravi S, Si-Tahar M, Chignard M, et al. (2007) The role of flagellin versus motility in acute lung disease caused by Pseudomonas aeruginosa. J Infect Dis 196: 289-296.
18. Matsumoto K (2004) Role of bacterial proteases in pseudomonal and serratial keratitis. Biol Chem 385: 1007-1016.
19. Azghani AO, Kondepudi AY, Johnson AR (1992) Interaction of Pseudomonas aeruginosa with human lung fibroblasts: role of bacterial elastase. Am J Respir Cell Mol Biol 6: 652-657.
20. Parmely M, Gale A, Clabaugh M, Horvat R, Zhou WW (1990) Proteolytic inactivation of cytokines by Pseudomonas aeruginosa. Infect Immun 58: 3009-3014.
21. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog 2: e56.
22. Rooijakkers SH, van Kessel KP, van Strijp JA (2005) Staphylococcal innate immune evasion. Trends Microbiol 13: 596-601.

23. Foster TJ (2005) Immune evasion by staphylococci. Nat Rev Microbiol 3: 948-958.
24. Chavakis T, Preissner KT, Herrmann M (2007) The anti-inflammatory activities of Staphylococcus aureus. Trends Immunol 28: 408-418.
25. de Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, et al. (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 329: 953-955.
26. Hege T, Feltzer RE, Gray RD, Baumann U (2001) Crystal structure of a complex between Pseudomonas aeruginosa alkaline protease and its cognate inhibitor: inhibition by a zinc-NH2 coordinative bond. J Biol Chem 276: 35087-35092.
27. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, et al. (2000) Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 406: 959-964.
28. Feltzer RE, Gray RD, Dean WL, Pierce WM, Jr. (2000) Alkaline proteinase inhibitor of Pseudomonas aeruginosa. Interaction of native and N-terminally truncated inhibitor proteins with Pseudomonas metalloproteinases. J Biol Chem 275: 21002-21009.
29. Caballero AR, Moreau JM, Engel LS, Marquart ME, Hill JM, et al. (2001) Pseudomonas aeruginosa protease IV enzyme assays and comparison to other Pseudomonas proteases. Anal Biochem 290: 330-337.
30. Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18: 265-276.
31. Gomez-Gomez L, Bauer Z, Boller T (2001) Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell 13: 1155-1163.
32. Fan LM, Zhao Z, Assmann SM (2004) Guard cells: a dynamic signaling model. Curr Opin Plant Biol 7: 537-546.
33. Melotto M, Underwood W, Koczan J, Nomura K, He SY (2006) Plant stomata function in innate immunity against bacterial invasion. Cell 126: 969-980.
34. Yonekura K, Maki-Yonekura S, Namba K (2003) Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. Nature 424: 643-650.
35. Koller B, Kappler M, Latzin P, Gaggar A, Schreiner M, et al. (2008) TLR expression on neutrophils at the pulmonary site of infection: TLR1/TLR2-mediated up-regulation of TLR5 expression in cystic fibrosis lung disease. J Immunol 181: 2753-2763.
36. Samakovlis C, Asling B, Boman HG, Gateff E, Hultmark D (1992) In vitro induction of cecropin genes--an immune response in a Drosophila blood cell line. Biochem Biophys Res Commun 188: 1169-1175.
37. Zeng W, He SY (2010) A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to Pseudomonas syringae pv tomato DC3000 in Arabidopsis. Plant Physiol 153: 1188-1198.
38. Stocker W, Grams F, Baumann U, Reinemer P, Gomis-Ruth FX, et al. (1995) The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci 4: 823-840.
39. Guzzo J, Duong F, Wandersman C, Murgier M, Lazdunski A (1991) The secretion genes of Pseudomonas aeruginosa alkaline protease are functionally related to those of Erwinia chrysanthemi proteases and Escherichia coli alpha-haemolysin. Mol Microbiol 5: 447-453.
40. Bestebroer J, Poppelier MJ, Ulfman LH, Lenting PJ, Denis CV, et al. (2007) Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 109: 2936-2943.
41. de Haas CJ, Veldkamp KE, Peschel A, Weerkamp F, Van Wamel WJ, et al. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. J Exp Med 199: 687-695.
42. Haas D, Holloway BW (1976) R factor variants with enhanced sex factor activity in Pseudomonas aeruginosa. Mol Gen Genet 144: 243-251.
43. Nabel G, Baltimore D (1987) An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326: 711-713.
44. Walasek P, Honek JF (2005) Nonnatural amino acid incorporation into the methionine 214 position of the

metzincin Pseudomonas aeruginosa alkaline protease. BMC Biochem 6: 21.
45. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, et al. (2003) Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 100: 14339-14344.
46. Pozo MJ, Van Der Ent S, Van Loon LC, Pieterse CM (2008) Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in Arabidopsis thaliana. New Phytol 180: 511-523.
47. Van der Ent S, Verhagen BW, Van Doorn R, Bakker D, Verlaan MG, et al. (2008) MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. Plant Physiol 146: 1293-1304.

Supplementary Figures

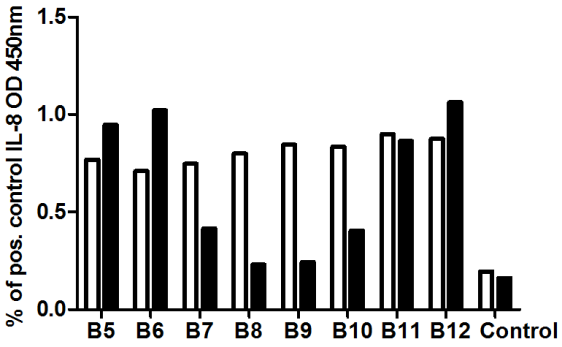

Figure S1. Fractionated *P. aeruginosa* supernatant does not inhibit IL-8 production of HEK/TLR4 cells.
P. aeruginosa supernatant was purified by ion-exchange chromatography and concentrated before gel filtration. Proteins were separated by size (Superdex 75) and 0.5 ml fractions were collected. HEK/TLR4 (white bars) and HEK/TLR5 (black bars) cells were pretreated with gel filtration fractions B5-B12 (10-fold diluted) for 30 minutes and subsequently challenged with 1 ng/ml LPS or flagellin, respectively. After 6 h stimulation cell culture supernatant was harvested and IL-8 concentration was determined by ELISA.

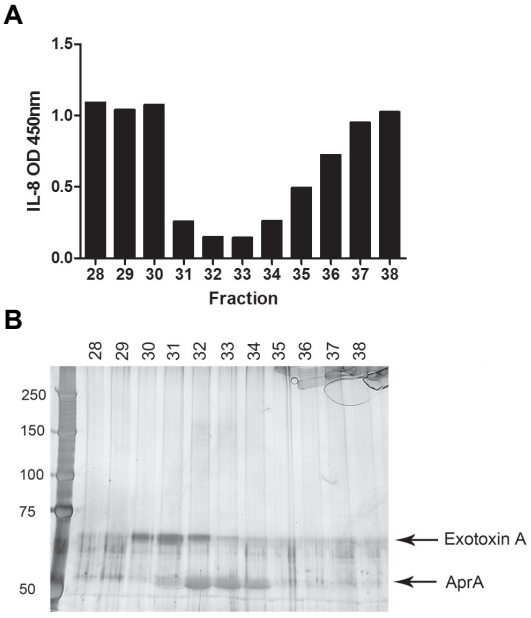

Figure S2. Identification of TLR5 inhibitory protein.
P. aeruginosa supernatant was separated by anion exchange (Q sepharose XL) and size exclusion chromatography (Superdex 75). (A) HEK/TLR5 cells were incubated with fractions after size exclusion chromatography for 30 min at 37°C, and subsequently challenged with 1 ng/ml flagellin for 6 h at 37°C. IL-8 levels were determined in the cell culture supernatant by ELISA. (B) Fractions were analyzed with SDS-PAGE and Coomassie staining.

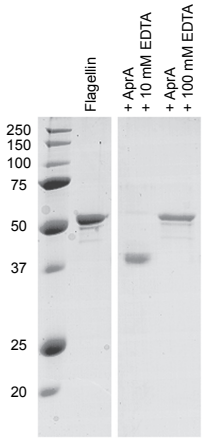

Figure S3. EDTA blocks AprA-mediated flagellin cleavage.
Flagellin (250 µg/ml) and AprA (1 µg/ml) were incubated in the presence of 10 mM or 100 mM EDTA for 60 min at 37°C. Flagellin degradation was analyzed by SDS-PAGE and Coomassie staining.

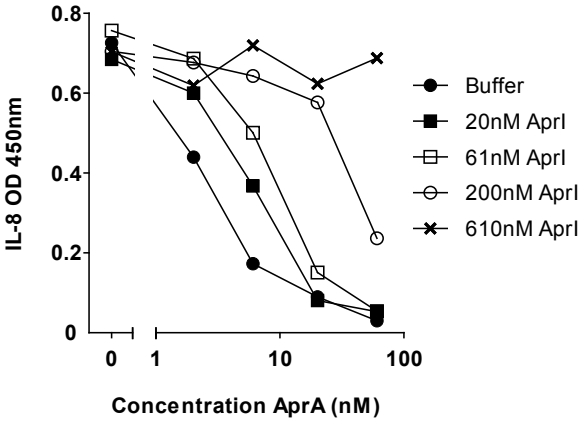

Figure S4. Dose-dependent inhibition of AprA by AprI.
HEK/TLR5 cells were challenged with 1 ng/ml *S. typhimurium* flagellin in the presence of an increasing concentration AprA premixed with AprI at 0, 20, 61, 200 or 610 nM. After six hours IL-8 production was measured by ELISA.

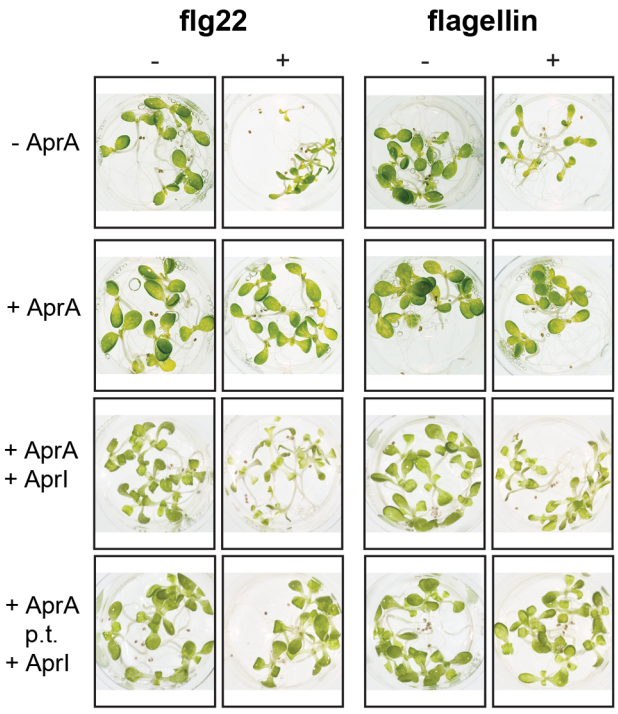

Figure S5. AprA prevents flagellin recognition in Arabidopsis.
A. thaliana La-*er* seedlings were incubated or not with 500 nM flg22 or *P. aeruginosa* flagellin that was preincubated with 3 µg/ml AprA when indicated. After treatment, seedlings were grown axenically for 10 days in MS medium and subsequently photographed. In the 3rd row panels AprI was added before AprA treatment and in the bottom panels post AprA treatment (p.t.) of flagellin.

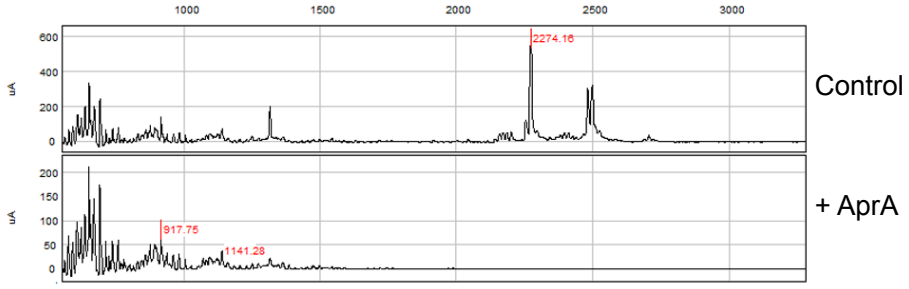

Figure S6. SELDI-TOF analysis of flg22 cleavage by AprA.
Flg22 (50 µM) was incubated with buffer (upper panel) or 3 µg/ml AprA (bottom panel) for 1 h at 37°C. For SELDI-TOF analysis untreated and AprA-treated peptide was diluted 250 times (200 nM) and spotted on a NP-20 array. Results between 500 Da and 2500 Da are shown, the 2274 Da peak represents flg22.

3

Pseudomonas aeruginosa Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways

Bart W. Bardoel*, Alexander J. Laarman*, Maartje Ruyken, Job Fernie, Fin J. Milder, Jos A.G. van Strijp, and Suzan H.M. Rooijakkers

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

*Both authors contributed equally

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Abstract

The complement system rapidly detects and kills Gram-negative bacteria and supports bacterial killing by phagocytes. However, bacterial pathogens exploit several strategies to evade detection by the complement system. The Alkaline protease (AprA) of *P. aeruginosa* has been associated with bacterial virulence and is known to interfere with complement-mediated lysis of erythrocytes, but its exact role in bacterial complement escape is unknown. In this study, we analyzed how AprA interferes with complement activation and whether it can block complement-dependent neutrophil functions. We find that AprA potently blocks phagocytosis and killing of Pseudomonas by human neutrophils. Furthermore, AprA inhibits opsonization of bacteria with C3b and the formation of the chemotactic agent C5a. AprA specifically blocks C3b deposition via the classical and lectin pathways while the alternative pathway is not affected. Serum degradation assays revealed that AprA degrades both human C1s and C2. However, repletion assays demonstrated that the mechanism of action for complement inhibition is cleavage of C2.

In summary, we show that *P. aeruginosa* AprA interferes with classical and lectin pathway mediated complement activation via cleavage of C2.

Introduction

The innate immune system rapidly detects and kills invading bacteria via different mechanisms, such as Toll-like receptors (TLR) and the complement system. TLRs are expressed on immune cells and detect an enormous variety of microorganisms via recognition of highly conserved microbial molecular patterns, such as flagellin via TLR5 and LPS via TLR4 [1]. Upon ligand binding, TLRs trigger an intracellular signalling cascade that leads to production of pro-inflammatory cytokines and activation of phagocytes. The complement system is a proteolytic cascade of plasma proteins, which results in direct killing of certain microorganisms and efficient bacterial recognition by phagocytes. The complement system consists of three distinguished pathways of which the classical (CP) and lectin pathway (LP) are important for recognition of bacteria, while the alternative pathway (AP) serves as and amplification loop. The CP and LP are initiated by binding of C1q and Mannose Binding Lectin (MBL) to bacterium-bound antibodies or bacterial sugars respectively [2]. The C1q- and MBL-associated proteases (C1s and MASP2) cleave the complement molecules C4 and C2 to induce formation of the C3 convertase complex (C4b2a). Cleavage of C3 by convertases is critical to opsonisation of the bacterial surface with C3b, which is recognized by complement receptors on neutrophils and supports phagocytosis and killing. Further downstream in the cascade, formation of C5 convertases and cleavage of C5 results in generation of the potent anaphylatoxin C5a and the formation of C5b-9, the membrane attack complex (MAC) that directly kills Gram-negative bacteria [3].

The Gram-negative bacterium *Pseudomonas aeruginosa* lives in water and soil and acts as an opportunistic pathogen in humans and plants. In healthy individuals, *P. aeruginosa* is efficiently killed via the innate immune system; however it causes chronic infections in immunocompromised patients. For instance, cystic fibrosis patients suffer from chronic infections caused by *P. aeruginosa* that cannot be eradicated after colonization. Strains isolated from cystic fibrosis patients rapidly adapt to the environment in the lung, resulting in dramatic genetic and morphological changes [4]. Infection by *P. aeruginosa* is complicated by its inherent resistance to several classes of antibiotics and acquisition of resistance genes via mobile genetic elements [5]. In addition, biofilm formation enhances the resistance to antibiotics in the cystic fibrosis lung [6]. Remarkably, the strains isolated from CF patients are generally sensitive to direct complement killing, which is different from strains isolated from non-CF patients. These serum-resistant strains consume more C5b-9 complement components and have less C3b deposited on their surface [7].

Bacterial survival in the host is accompanied by the acquisition of different immune evasive strategies. These strategies involve alteration of the bacterial surface such as production of a capsule or immune-modulating membrane proteins, or secretion of soluble immune evasion proteins such as proteolytic factors. *P. aeruginosa* secretes a number of toxins and virulence factors like elastase and alkaline protease. Elastase cleaves a wide variety of host proteins such as collagen, IgG and complement proteins [8]. Alkaline protease (AprA) is a 50 kD zinc metalloprotease that degrades several components of the host immune system such as complement C1q and C3 [9] or cytokines like IFN-gamma and TNF-alpha [10]. In addition, AprA prevents TLR5 activation via the cleavage of monomeric flagellin (Chapter 2). The AprA is secreted via its own type I secretion system, that is encoded by three genes upstream of the *aprA* gene. *P. aeruginosa* also encodes a highly specific inhibitor of alkaline protease named AprI, which is translocated to the periplasmic space according to its signal sequence.

The role of AprA in Pseudomonal virulence has been illustrated in a Drosophila infection model [11]. AprA contributes to the persistence of *P. entomophila* infection, and protects against antimicrobial peptides produced by the innate immune system of *Drosophila*.
In a previous study Hong et al. showed that AprA cleaves complement components C1q and C3 and inhibits lysis of sheep erythrocytes via the membrane attack complex [9]. However, since *P. aeruginosa* is generally resistant to direct lysis via the membrane attack complex, we wondered whether cleavage of complement proteins by AprA contributes to Pseudomonal immune evasion. We find that AprA blocks various important complement functions hampering the bacterial clearance by human neutrophils. Furthermore, by using biological assays with more physiological relevant protease concentrations, we uncovered that AprA specifically blocks the classical and lectin pathways and predominantly cleaves complement protein C2.

Materials and methods

Proteins, sera and bacterial strains

C3 was purified from human plasma as described [12]. The purified components C1, C2, and C4 were purchased from Quidel. C1s was obtained from R&D and C2-depleted serum was purchased from Sigma. Normal human serum was obtained from healthy volunteers, who gave informed consent. AprA and AprI of *P. aeruginosa* PAO1 were expressed and isolated from *E. coli* as described (Chapter 2). Bacterial strains: *P. aeruginosa* PAO1, *E. coli* K-12 and GFP-PAO1 [13] (kindly provided by J.M. Beekman)

CH50

The classical pathway hemolytic assay was performed as previously described [14] with minor modifications. Serum was pre-incubated with 20, 66 and 200 nM AprA or 200 nM AprA plus 1000 nM AprI for 30 min at 37°C. Subsequently, opsonized sheep erythrocytes (anti-sheep IgM) were incubated with AprA-treated serum in veronal buffered saline containing 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$. After 30 min at 37°C samples were centrifuged, and the absorbance of the supernatants at 405 nm was measured.

E. coli killing

E. coli K12 was cultured in LB medium overnight at 37°C. The next day, the overnight culture was diluted 20 times in LB and grown until an OD_{660nm} of 0.5. Bacteria were washed with RPMI supplemented with 0.05% HSA. Human pooled serum (HPS) was incubated with different concentrations AprA with or without 1000 nM AprI for 30 min at 37°C in RPMI-HSA. Subsequently, bacteria were added ($1x10^5$/ml) and incubated for 1 h at 37°C. Colony forming units (CFU) were determined by plating serial dilutions on Tryptic soy agar + 5% sheep blood (Oxoid).

Phagocytosis and killing

Phagocytosis assays were performed as described [15]. In short, serum was pre-incubated with buffer, 200 nM AprA, or 200 nM AprA plus 1000 nM AprI in RPMI containing 0.1% HSA for 30 min at 37°C. Then, $2.5x10^5$ freshly isolated human neutrophils and $2.5x10^6$ GFP-labeled, heat-killed *P. aeruginosa* were added and incubated for 15 min at 37°C while

shaking at 600 rpm. The reaction was stopped by adding 1% ice-cold paraformaldehyde in RPMI containing 0.1% HSA. Phagocytosis was analyzed using the FACSCalibur (Becton Dickinson). In another experiment, different concentrations of AprA were incubated with 5 % serum in RPMI containing 0.1% HSA for 30 min at 37°C. The IC50 was calculated with the formula y=-0.2793x + 85.536.

For neutrophil killing assays, *P. aeruginosa* was grown to an OD_{660} of 0.5 in LB and subsequently washed in RPMI containing 0.1% HSA. Then, 5% serum was pre-incubated with buffer, 200 nM AprA, or 200 nM AprA plus 1000 nM AprI for 30 min at 37°C. Subsequently, 2.5 x 10^5 *P. aeruginosa* and 8.5 x 10^6 neutrophils were added and incubated at 37°C. At different time points a sample was taken and neutrophils were lysed with Milli-Q. Surviving bacteria were enumerated by plating serial dilutions on LB agar (Oxoid).

C3b deposition on P. aeruginosa

An overnight culture of *P. aeruginosa* strain PA01-GFP in LB was washed in veronal buffered saline containing 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$ and 0.1% BSA. Serum was pre-incubated with different concentrations AprA with or without AprI for 30 min at 37°C. Then, 2.5 x 10^6 bacteria were incubated with the pre-incubated serum for 30 min while shaking at 900 rpm. Bacteria were washed with PBS with 0.1% BSA. C3b deposition was detected using mouse anti-human C3b antibodies (WM-1, ATCC) and APC-conjugated goat anti-mouse IgG (Protos). Fluorescence of 10,000 bacteria was measured by flow cytometry.

C5a analysis

An overnight culture of *P. aeruginosa* strain PAO1 in LB was washed in RPMI with 0.1% HSA. 10% Serum and 200 nM AprA or 200 nM AprA plus 1000 nM AprI were pre-incubated for 30 min at 37°C and subsequently incubated with 5 x 10^7 bacteria at 37°C for 30 min while shaking at 600 rpm. Bacteria were centrifuged and C5a was detected in collected supernatants by calcium mobilization: 10-fold diluted supernatants were added to 5 x10^4 Fluo-4-AM labeled U937-C5a receptor cells (U937-C5aR; a generous gift from Prof. Eric Prossnitz, University of New Mexico, Albuquerque, USA) and the increase of intracellular calcium was measured by flow cytometry.

Complement assays

Complement ELISAs were performed as described [16] with modifications. ELISA plates (Nunc, Maxisorb) were coated overnight with 20 µg/ml LPS (*Salmonella enteritidis,* Sigma), 3 µg/ml IgM (Quidel) or 10 µg/ml mannan (*Saccharomyces cerevisiae,* Sigma) in 0.1 M sodium carbonate buffer pH 9.6. Plates were blocked with 4% BSA in PBS with 0.05% Tween for 1 h at 37°C. For the CP and LP, samples were diluted in veronal buffered saline containing 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$, 0.1% gelatin, and 0.05% Tween. For the AP, samples were diluted in veronal buffered saline containing 5 mM $MgCl_2$, 10 mM EGTA, 0.1% gelatin, and 0.05% Tween. Serum or C2 depleted serum was mixed with different concentrations AprA or AprA together with AprI and subsequently added to the plates for 1 h at 37°C. Deposited C3b and C4b were detected using antibodies against C3d (WM-1 DIG-labeled) and C4d respectively (Quidel), followed by peroxidase (PO)-conjugated sheep anti-DIG or a goat anti–mouse IgG (Southern Biotechnology) respectively. For the repletion experiment IgM-coated plates were used and the assay was performed as described above. In short, 1

% serum was pre-incubated with 200 nM AprA for 30 min at 37°C. Then, 1000 nM AprI was added together with C1 (2 nM), C1s (3 nM), C2 (2 nM), C3 (67 nM), or C4 (20 nM) and the mixtures were added to the plate for 1 h at 37°C. C3b deposition was detected as described above.

C2 cleavage

C2 was incubated with different concentration of AprA for 30 min at 37°C in veronal buffered saline containing 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$. Proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. Cleavage products were visualized with 0.1% Coomassie blue in 50% methanol, excised and analyzed by N-terminal sequencing (Alphalyse).

Western blotting

PAO1 was cultured overnight in LB and subsequently diluted 10 times in IMDM (Invitrogen). Bacterial supernatant was collected after 2, 4 and 6 hours. The presence of AprA in supernatant of *P. aeruginosa* was analyzed by Western blotting using a protein G-purified polyclonal rabbit anti-AprA (Genscript), followed by a goat anti-rabbit IgG antibody (Southern Biotech).

Analysis of complement factor cleavage in serum was performed by Western blotting. 3% serum was incubated with different concentrations AprA at 37°C in PBS. The reaction was stopped by adding Laemmli sample buffer containing DTT. All samples were subjected to SDS-PAGE and blotted onto a PVDF membrane. After blocking with 4% skimmed milk in PBS containing 0.1% Tween, C1s, C2, C3, C4 and fB was detected by a mouse anti-human C1s antibody (Quidel), goat anti-human fB, C2 (both Quidel), and C3 antibody (Protos), and chicken anti-human C4 (Quidel), respectively, followed by a donkey anti-goat IgG antibody (Jackson) diluted in PBS with 0.1% Tween and 1% skimmed milk. ECL (GE Healthcare) was used for final signal detection.

Results

Physiological AprA levels inhibit complement-mediated lysis

To study the functional relevance of AprA secretion by *P. aeruginosa* on the complement system, we first estimated AprA concentrations in culture supernatants. Supernatants were collected at different time points and analyzed for AprA expression by Western blotting using an antibody against AprA (Fig. 1A). The levels of AprA in the supernatant were semi-quantified by comparison with fixed concentrations of recombinant AprA. The AprA concentration at 6 hours of culture was estimated between 22-66 nM (1-3 µg/ml). As AprA has been described to inhibit complement-dependent hemolysis via the membrane attack complex, we repeated the assay to confirm AprA activity. AprA inhibited the lysis of antibody-sensitized sheep erythrocytes, a classical pathway-meditated hemolysis assay, which was restored in the presence of AprI (Fig. 1B). Furthermore, we tested different concentration of AprA in a serum-killing assay using *E. coli* as a model. In contrast to *P. aeruginosa* strain PAO1, *E. coli* K-12 is not serum-resistant and is lysed at low serum concentrations due to incorporation of the lytic MAC. Indeed, AprA inhibited the complement-dependent lysis with an estimated IC50 of 30 nM, which is in a similar concentration range as detected in the supernatant (Fig. 1C). The natural inhibitor AprI blocks proteolytic activity of AprA [17]

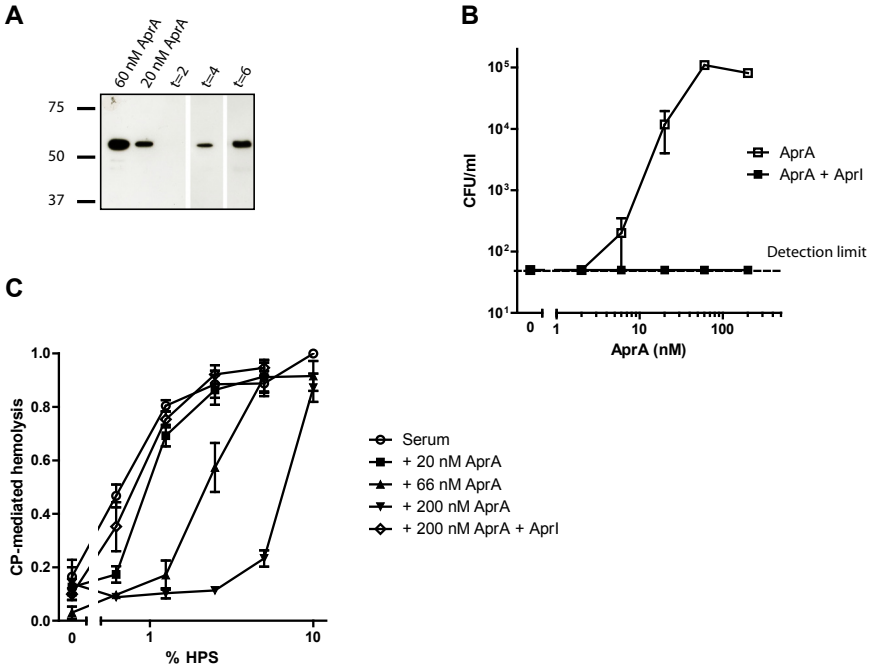

Figure 1. Secreted AprA concentration sufficient to inhibit complement activity
(A) Overnight culture of *P. aeruginosa* (PAO1) was diluted ten times in IMDM. After 2, 4 and 6 hours supernatant was collected and analyzed for AprA production by western blot using a polyclonal antibody against AprA. (B) Serum was preincubated with different concentrations AprA (nM) or together with 1000 nM AprI for 30 min at 37°C. Subsequently, *E. coli* K12 (1x10^5 CFU/ml) in RPMI-HSA was added to treated serum for 1 h at 37°C. Number of surviving bacteria per ml (CFU/ml) was determined by serial dilutions. Data represent mean ± SEM of three independent experiments. (C) Serum was preincubated with AprA with or without AprI (1000 nM) for 30 min at 37°C. Treated serum was mixed with opsonized sheep erythrocytes and incubated for 1 h at 37°C. Erythrocytes were pelleted and optical density of supernatant at 405 nm was measured. Data are expressed as relative lysis compared to lysed erythrocytes and represent mean ± SEM of three independent experiments.

thereby allowing complement-dependent lysis of *E. coli*. Thus, AprA blocks complement-dependent lysis in a concentration range similar to AprA levels in *P. aeruginosa* supernatant.

AprA inhibits complement-dependent neutrophil functions important for P. aeruginosa killing

Since many *P. aeruginosa* strains are resistant to direct complement lysis [18], we investigated whether AprA facilitates resistance to other complement processes that are critical to neutrophil functioning. To study whether AprA blocks complement-dependent phagocytosis, we incubated GFP-labeled *P. aeruginosa* with human neutrophils in the presence of human serum and AprA. AprA blocked the phagocytosis compared to buffer or AprA supplemented with AprI (Fig. 2A), and its activity is dose-dependent (Fig. 2B). The IC50 of AprA is 84 nM, which is in the relevant range produced by *P. aeruginosa*. Incubation of *P. aeruginosa* with serum results in the deposition of C3b molecules on the bacterial surface, which are recognized by complement receptors on phagocytes. To study whether AprA inhibits the C3b deposition, we incubated bacteria with serum and detected surface-bound C3b using specific antibodies and flow cytometry. AprA inhibited C3b deposition on

Pseudomonas at comparable concentrations as observed for phagocytosis of *P. aeruginosa* (Fig. 2C). Further along the complement cascade, C5 is cleaved into the potent anaphylatoxin C5a that is recognized by the C5a receptor on neutrophils. Activation of the C5a receptor induces intracellular calcium mobilization and chemotaxis of the neutrophil to the site of inflammation. We incubated *P. aeruginosa* with serum and studied the release of C5a into the supernatant by measuring the supernatant-induced calcium mobilization on Fluo-3-AM labeled U937 cells transfected with the C5a receptor. When bacteria were incubated with AprA, supernatants could not induce calcium mobilization, meaning that AprA inhibited C5a formation (Fig. 2D). To verify that AprA also inhibits the killing of *P. aeruginosa* by neutrophils, we incubated neutrophils with bacteria and serum and determined the percentage of survival after 15 min. After 15 min 80% of the bacteria were killed. AprA inhibited the killing of *P. aeruginosa*, but not completely, showing a killing of 40% after 15 min. This effect was restored in the presence of AprI. In summary, AprA inhibits all complement dependent effector functions, which allows *P. aeruginosa* to escape neutrophil killing.

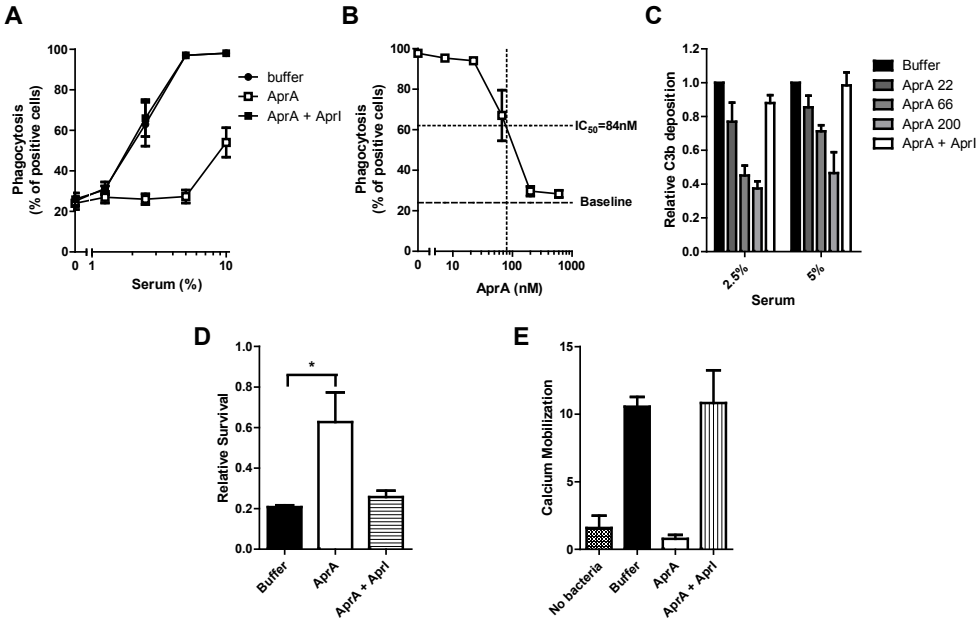

Figure 2. AprA inhibits complement-dependent effector functions important for *P. aeruginosa*
(A and B), Phagocytosis of *P. aeruginosa* PAO1-GFP by human neutrophils in the presence of human serum and AprA. (A), AprA blocks phagocytosis of bacteria in human serum. (B), Dose-dependent inhibition of phagocytosis by AprA in 5% serum. (C), Inhibition of C3b deposition on *P. aeruginosa* by AprA. Serum was preincubated with different concentrations of AprA (nM) and mixed with bacteria. Deposition of C3b on the bacterial surface was measured by flow cytometry. (D), Inhibition of C5a release by AprA. Serum (10%) was preincubated with AprA with or without AprI and subsequently incubated with bacteria. Release of C5a in bacterial supernatants was measured by a calcium mobilization assay using U937-C5aR cells. (E), AprA blocks killing of *P. aeruginosa* by human neutrophils in 5% serum (AprA at 200 nM and AprI at 1000 nM). All figures represent mean ± SEM of three independent experiments. For (D), the relative calcium mobilization was calculated by dividing the fluorescence after stimulation by the baseline fluorescence. For (E), the relative survival was calculated by dividing the number of CFUs by the number of CFUs at time point zero.

AprA inhibits complement activation via the classical and lectin pathways

To further study the effect of AprA on the complement system, we dissected the individual pathways using pathway-specific complement ELISA's [16]. The classical (CP), lectin (LP) and alternative pathway (AP) were specifically induced in the presence of serum using the coatings IgM, mannan, and LPS respectively. The level of C3b deposition was detected with specific antibodies. AprA strongly inhibited the C3b deposition of the CP (Fig. 3A) and LP (Fig. 3B) at all tested serum concentrations. Inhibition of the AP was not observed at AprA concentrations up to 66 nM (Fig. 3C). As mentioned above, C3b deposition via the CP and LP is mediated by C3 convertases, bimolecular complexes that consist of the protease C2a loosely attached to surface-bound C4b. C4b and C2a are formed by cleavage of C4 and C2 by the C1 or MBL-MASP2 complexes. To study at what level AprA affects these pathways, we also analyzed the C4b deposition. In contrast to C3b deposition, AprA did not block C4b deposition in the CP and LP (Fig. 3D and 3E). This indicates that AprA specifically blocks the CP and LP upstream of C3 and downstream of C4 activation in the complement cascade.

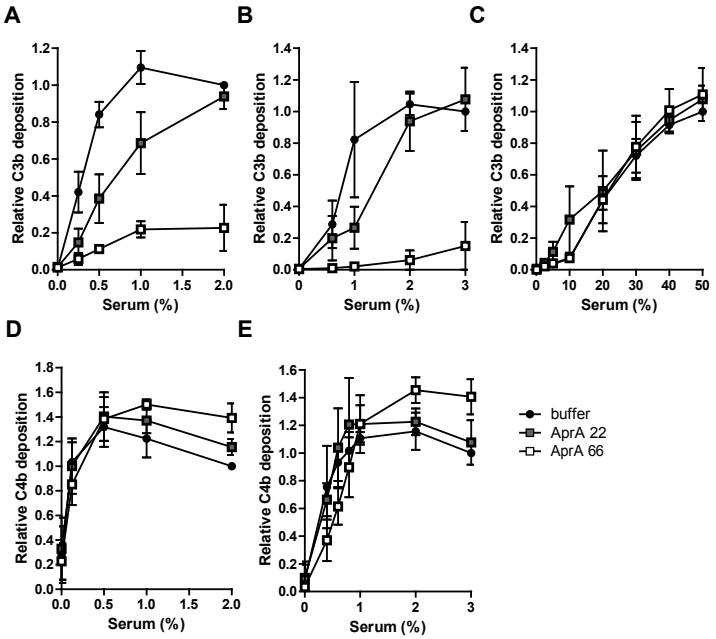

Figure 3. AprA inhibits complement activation via the classical and lectin pathways
Serum was incubated with different concentrations of AprA, and complement activation via the CP, LP and AP was determined via ELISA. AprA prevents C3b deposition via the CP (A) and the LP (B), but not the AP (C). No inhibition of C4b deposition was observed via the CP (D) and LP (E). All figures represent mean ± SEM of three independent experiments.

AprA depletes the limiting factor C2

Many complement factors are involved in the CP and LP that induce C3b deposition. However, only two factors (C2 and C3) are involved after C4b deposition and before C3b deposition. To identify the critical factor that is cleaved by AprA, we incubated human serum with AprA for 30 min at 37°C. The complement factors C2, C3 and C4 were analyzed by western blotting.

As control C1s, important for C4b deposition of the CP, and fB, involved in C3 convertase formation of the AP, were detected. AprA efficiently cleaved C2, while C3, C4 and fB were not affected (Fig. 4A). In addition, cleavage of C1s by AprA was observed, however this was less efficient than C2 cleavage. We used the CP complement ELISA to discriminate the limiting factor that was cleaved by AprA. First we incubated serum with AprA and inactivated the protease by adding the irreversible inhibitor AprI. Then, treated serum was used in the CP ELISA supplemented with the different purified complement factors. Only the serum that was supplemented with purified C2 could restore the complement activation, while AprA-treated serum supplemented with C1, C1s, C3 showed no activity (Fig. 4B). Thus, even though AprA also cleaves C1s, the cleavage of C2 determines inhibition of complement activation.

AprA cleaves C2

C2 is a 100kDa protein that consists of three Complement Control Protein regions (CCP1-3), a von Willebrand Factor A (vWFA) domain and a peptidase domain. The cleavage of C2 by its natural proteases C1s and MASP2 results in formation of C2b (CCP1-3) and C2a (VWFA and peptidase domain) (Fig. 5B and 5C). For the formation of a functional C3 convertase (C4b2a), full-length C2 first binds to C4b and is subsequently cleaved. C2b is then released, while C2a stays attached to C4b. The C4b2a convertase has a very short half-life due to the irreversible dissociation of C2a. C2 cleavage by AprA was further analyzed by SDS-PAGE and Coomassie staining. After incubating of purified C2 with AprA, we observed three cleavage products of respectively ~75kDa, ~40kDa and ~30kDa, which were send for N-terminal sequencing (Fig. 5A). Cleavage product 1 starts with NH_2-IQIQRS, which is only one amino acid different from C2a (starts with NH_2-KIQIQR). At higher protease concentrations, an increase in cleavage

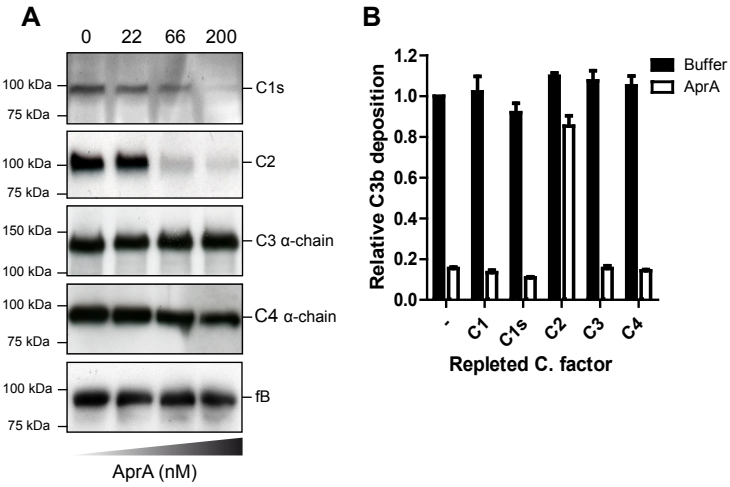

Figure 4. AprA depletes the limiting factor C2
(A) AprA degrades C2 and C1s. Different concentrations of AprA were incubated with 3% serum for 30 min at 37°C. C1s, C2, C3, C4 and fB were detected by western blotting. (B) C2 is the limiting factor. Serum (1%) was preincubated with 200 nM AprA for 30 min at 37°C. Then, 0.2 µM AprI was added to stop the AprA cleavage, together with C1, C1s, C2, C3, or C4. Complement activation via the CP was determined by ELISA by detecting C3b deposition.

product 2 is observed meaning that AprA secondly cleaves between the VWFA and peptidase domain. Next to C2a, AprA cleaves C2b between CCP1 and CCP2. These data indicate that AprA efficiently interferes with CP and LP activation via the degradation of C2.

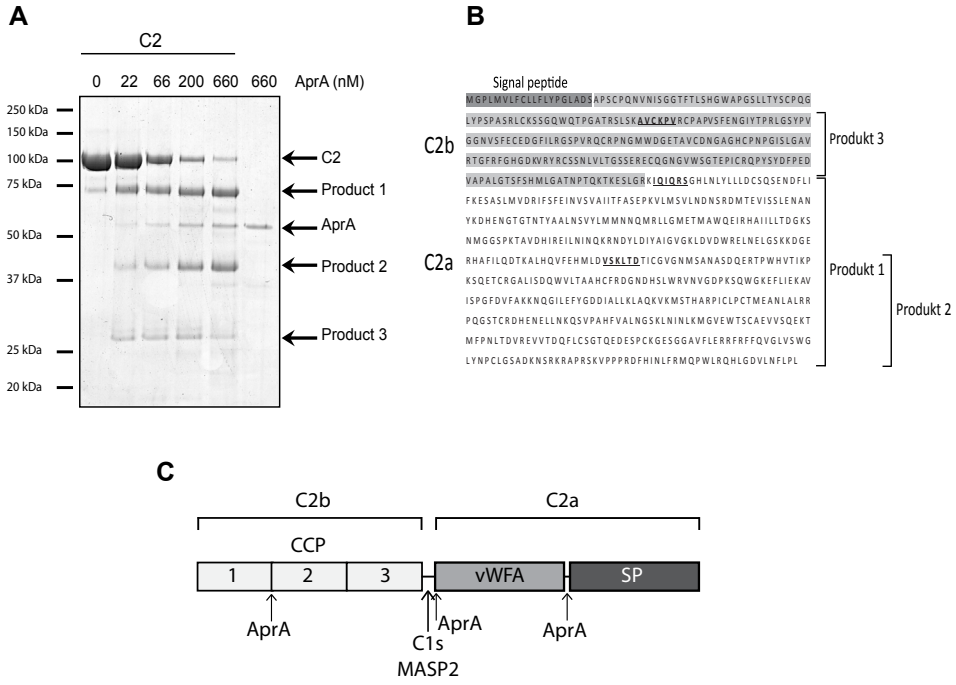

Figure 5. AprA cleaves C2
(A) C2 cleavage by AprA. Purified C2 was incubated with different concentrations AprA and cleavage was analyzed by SDS-PAGE and Coomassie staining. Cleavage product 1, 2, and 3 were sent for N-terminal sequencing. (B) Sequence of human C2 with the obtained N-terminal sequences of cleavage products 1, 2, and 3 underlined. (C) Schematic representation of C2. Indicated are the domains and the cleavage site for C1s, MASP2,and AprA

Discussion

Bacterial pathogens adopt many strategies to counteract the redundant attack of the human host defense. For instance, bacteria secrete many proteins that specifically interact with the complement system, which can either be proteolytic or steric interactions [19,20]. In the defense against Gram-negative bacteria, the complement system directly destroys cells by inserting the pore-forming MAC into the bacterial membrane [21]. Whereas all Gram-positive bacteria are protected from MAC lysis due to their thick peptidoglycan layer, some Gram-negative bacteria have also become resistant to direct complement attack by altering their surface properties [22]. The Gram-negative pathogen *P. aeruginosa* does this by lengthening the O-antigen side chains of lipopolysaccharide in the outer membrane [18]. However, resisting the MAC is likely not sufficient for Gram-negative pathogens to fully overcome complement-mediated immune clearance. Complement also labels these

bacteria with C3b to support phagocytic uptake and generates C5a to attract phagocytes to the site of infection. *In vivo* studies have shown that complement receptor 3 [23,24] and the C5a receptor [25,26,27] on neutrophils are important for protection against *P. aeruginosa* lung infection. Here we show that *P. aeruginosa* also inhibits other steps in the complement cascade by secretion of the metalloprotease AprA. AprA blocks classical and lectin pathway activation and thereby prevents C3b-dependent uptake of *Pseudomonas* by neutrophils and C5a-dependent neutrophil activation. Previously it was shown that AprA inhibits MAC-dependent lysis of erythrocytes [9]. Now we indicate how AprA contributes to pseudomonal resistance against complement effector functions relevant to host clearance of this bacterium. Furthermore, we identify the molecular mechanism to be cleavage of C2. AprA is the first bacterial protease that cleaves C2. Since C2 concentrations in serum (0.25 μM) are low compared to other complement molecules (C3 at 6.8 μM, C4 at 2.1 μM), this molecule is a vulnerable target for proteolytic degradation. Interestingly, we find that AprA cleaves C2 very close to the cleavage site of its natural proteases C1s and MASP2. Strikingly, this is similar to the *Staphylococcus aureus* metalloprotease aureolysin, which cleaves the C3 molecule two amino acids apart from the natural protease cleavage site [28](. Likely, the natural cleavage site in these proteins is an accessible part of the protein that makes it vulnerable for cleavage by bacterial proteases. Figure 6 provides a schematic overview of how we think AprA blocks complement activation. The formation of the C4b2a convertase is a multistep process [29]. First, C2 binds surface-bound C4b and is subsequently cleaved into C2a forming the active convertase. Due to its short half-life, C2a is released from the complex and cannot re-associate with C4b. The fluid-phase cleavage of C2 by AprA will prevent formation of the pro-convertase C4bC2 and an active C4b2a complex. At present, it is not clear whether the additional cleavage sites of AprA in C2 (between CCP1 and CCP2, and between the VWFA and peptidase domain) are functionally relevant.

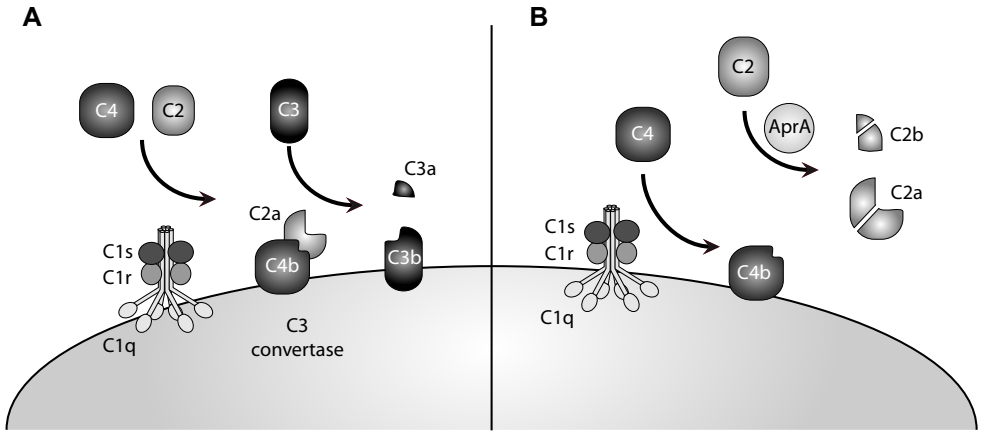

Figure 6. Schematic representation of the complement-inhibitory mechanism of AprA.
(A) In the classical pathway, fluid-phase C4 and C2 are cleaved by C1s (MASP2 for the lectin pathway) on the bacterial surface. This results in the formation of the C3 convertase (C4b2a), which cleaves C3 resulting in C3b deposition on the bacterial surface. (B) Complement activation in the presence of AprA. C2 is cleaved by AprA into fluid-phase C2a and C2b, subsequently further cleavage of both C2a and C2b occurs. Since fluid-phase C2a cannot bind to C4b, an active C3 convertase cannot be generated. In this way, AprA prevents C3b deposition on the bacterial surface and downstream complement effector functions.

Production of AprA by *P. aeruginosa* differs among strains and is highly dependent on culture conditions [30]. This is illustrated by the enhanced production of AprA by culturing in the presence of sputum from cystic fibrosis patients. [31]. *P. aeruginosa* produces detectable levels of AprA *in vivo* as measured in samples obtained from patients with corneal infections [32] and in sputum isolated from cystic fibrosis patients [33,34]. The observed *in vivo* concentrations tend to be in a different range than observed in *P. aeruginosa* overnight cultures and the concentrations used in our study. However, determination of AprA concentration *in vivo* is complicated by the fact that AprA is produced locally in the surrounding of the bacterium, and subsequently diluted or degraded by host proteases in clinical samples. In a rat pouch model only small amounts of injected recombinant AprA could be recovered after six hours [35], suggesting that production of AprA *in vivo* is a lot higher than measured. Here we show that AprA cleaves C1s and C2 in concentrations similar to levels secreted by *P. aeruginosa* in functionally relevant time incubations. However, increasing AprA concentrations or incubation times allows cleavage of more complement factors such as C3 and C4 (data not shown). A previous paper also showed that AprA cleaves C3 and C1q, but high concentrations of AprA (400 nM) and long incubation times (20 h) were used [9]. Our functional repletion assay (Fig. 4B) clearly showed that complement inhibition by AprA is exclusively mediated by the cleavage of C2. Furthermore, the cleavage of C2 is in line with our findings that AprA does not affect the alternative pathway, since activation of this route is not dependent on C2. The AprA concentrations used for cleavage of C2 were in a similar range as previous studies examining AprA cleavage of flagellin (Chapter 2) and other human immune proteins [8].

Thus, we show that AprA blocks activation of the classical and lectin pathway by degrading C2. Thereby *P. aeruginosa* evades complement-mediated phagocytosis and killing by neutrophils. Known other complement-modulating proteins of *P. aeruginosa* are: the intracellular elongation factor tuf binds factor H [36], Elastase degrades C3 [37] and Protease IV degrades C1q and C3 [38]. As observed for other bacterial pathogens [20], complement resistance factors in *P. aeruginosa* are probably as redundant as the complement system itself. Especially since *P. aeruginosa* strains from CF patients are sensitive to complement lysis [39,40], we expect this bacterium to have multiple molecules that counteract complement activation and enable bacterial survival in the human host.

Acknowledgements

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Reference List

1. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373-384.
2. Gasque P (2004) Complement: a unique innate immune sensor for danger signals. Mol Immunol 41: 1089-1098.
3. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, et al. (2009) The role of the anaphylatoxins in health and disease. Mol Immunol 46: 2753-2766.
4. Hassett DJ, Sutton MD, Schurr MJ, Herr AB, Caldwell CC, et al. (2009) Pseudomonas aeruginosa hypoxic or anaerobic biofilm infections within cystic fibrosis airways. Trends Microbiol 17: 130-138.
5. Kung VL, Ozer EA, Hauser AR (2010) The accessory genome of Pseudomonas aeruginosa. Microbiol Mol Biol Rev 74: 621-641.
6. Drenkard E, Ausubel FM (2002) Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 416: 740-743.
7. Schiller NL, Hatch RA, Joiner KA (1989) Complement activation and C3 binding by serum-sensitive and serum-resistant strains of Pseudomonas aeruginosa. Infect Immun 57: 1707-1713.
8. Matsumoto K (2004) Role of bacterial proteases in pseudomonal and serratial keratitis. Biol Chem 385: 1007-1016.
9. Hong YQ, Ghebrehiwet B (1992) Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol 62: 133-138.
10. Parmely M, Gale A, Clabaugh M, Horvat R, Zhou WW (1990) Proteolytic inactivation of cytokines by Pseudomonas aeruginosa. Infect Immun 58: 3009-3014.
11. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog 2: e56.
12. Rooijakkers SH, Wu J, Ruyken M, van Domselaar R, Planken KL, et al. (2009) Structural and functional implications of the alternative complement pathway C3 convertase stabilized by a staphylococcal inhibitor. Nat Immunol 10: 721-727.
13. Kuchma SL, Connolly JP, O'Toole GA (2005) A three-component regulatory system regulates biofilm maturation and type III secretion in Pseudomonas aeruginosa. J Bacteriol 187: 1441-1454.
14. Bestebroer J, Aerts PC, Rooijakkers SH, Pandey MK, Kohl J, et al. (2010) Functional basis for complement evasion by staphylococcal superantigen-like 7. Cell Microbiol 12: 1506-1516.
15. Rooijakkers SH, Ruyken M, Roos A, Daha MR, Presanis JS, et al. (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. Nat Immunol 6: 920-927.
16. Roos A, Bouwman LH, Munoz J, Zuiverloon T, Faber-Krol MC, et al. (2003) Functional characterization of the lectin pathway of complement in human serum. MolImmunol 39: 655-668.
17. Feltzer RE, Gray RD, Dean WL, Pierce WM, Jr. (2000) Alkaline proteinase inhibitor of Pseudomonas aeruginosa. Interaction of native and N-terminally truncated inhibitor proteins with Pseudomonas metalloproteinases. J Biol Chem 275: 21002-21009.
18. Hancock RE, Mutharia LM, Chan L, Darveau RP, Speert DP, et al. (1983) Pseudomonas aeruginosa isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect Immun 42: 170-177.
19. Potempa J, Pike RN (2009) Corruption of innate immunity by bacterial proteases. J Innate Immun 1: 70-87.
20. Laarman A, Milder F, van Strijp J, Rooijakkers S (2010) Complement inhibition by gram-positive pathogens: molecular mechanisms and therapeutic implications. J Mol Med 88: 115-120.
21. Hadders MA, Beringer DX, Gros P (2007) Structure of C8alpha-MACPF reveals mechanism of membrane attack in complement immune defense. Science 317: 1552-1554.
22. Nakamura S, Shchepetov M, Dalia AB, Clark SE, Murphy TF, et al. (2011) Molecular basis of increased serum

resistance among pulmonary isolates of non-typeable Haemophilus influenzae. PLoS Pathog 7: e1001247.
23. Gyetko MR, Sud S, Kendall T, Fuller JA, Newstead MW, et al. (2000) Urokinase receptor-deficient mice have impaired neutrophil recruitment in response to pulmonary Pseudomonas aeruginosa infection. J Immunol 165: 1513-1519.
24. Qin L, Quinlan WM, Doyle NA, Graham L, Sligh JE, et al. (1996) The roles of CD11/CD18 and ICAM-1 in acute Pseudomonas aeruginosa-induced pneumonia in mice. J Immunol 157: 5016-5021.
25. Cerquetti MC, Sordelli DO, Bellanti JA, Hooke AM (1986) Lung defenses against Pseudomonas aeruginosa in C5-deficient mice with different genetic backgrounds. Infect Immun 52: 853-857.
26. Hopken UE, Lu B, Gerard NP, Gerard C (1996) The C5a chemoattractant receptor mediates mucosal defence to infection. Nature 383: 86-89.
27. Larsen GL, Mitchell BC, Harper TB, Henson PM (1982) The pulmonary response of C5 sufficient and deficient mice to Pseudomonas aeruginosa. Am Rev Respir Dis 126: 306-311.
28. Laarman AJ, Ruyken M, Malone CL, van Strijp JA, Horswill AR, et al. (2011) Staphylococcus aureus Metalloprotease Aureolysin Cleaves Complement C3 To Mediate Immune Evasion. J Immunol.
29. Fujita T (2002) Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol 2: 346-353.
30. Cryz SJ, Iglewski BH (1980) Production of alkaline protease by Pseudomonas aeruginosa. J Clin Microbiol 12: 131-133.
31. Duan K, Surette MG (2007) Environmental regulation of Pseudomonas aeruginosa PAO1 Las and Rhl quorum-sensing systems. J Bacteriol 189: 4827-4836.
32. Kernacki KA, Hobden JA, Hazlett LD, Fridman R, Berk RS (1995) In vivo bacterial protease production during Pseudomonas aeruginosa corneal infection. Invest Ophthalmol Vis Sci 36: 1371-1378.
33. Jaffar-Bandjee MC, Lazdunski A, Bally M, Carrere J, Chazalette JP, et al. (1995) Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by Pseudomonas aeruginosa. J Clin Microbiol 33: 924-929.
34. Doring G, Obernesser HJ, Botzenhart K, Flehmig B, Hoiby N, et al. (1983) Proteases of Pseudomonas aeruginosa in patients with cystic fibrosis. J Infect Dis 147: 744-750.
35. Doring G, Dalhoff A, Vogel O, Brunner H, Droge U, et al. (1984) In vivo activity of proteases of Pseudomonas aeruginosa in a rat model. J Infect Dis 149: 532-537.
36. Kunert A, Losse J, Gruszin C, Huhn M, Kaendler K, et al. (2007) Immune evasion of the human pathogen Pseudomonas aeruginosa: elongation factor Tuf is a factor H and plasminogen binding protein. J Immunol 179: 2979-2988.
37. Schmidtchen A, Holst E, Tapper H, Bjorck L (2003) Elastase-producing Pseudomonas aeruginosa degrade plasma proteins and extracellular products of human skin and fibroblasts, and inhibit fibroblast growth. Microb Pathog 34: 47-55.
38. Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ (1998) Protease IV, a unique extracellular protease and virulence factor from Pseudomonas aeruginosa. J Biol Chem 273: 16792-16797.
39. Schiller NL, Alazard MJ, Borowski RS (1984) Serum sensitivity of a Pseudomonas aeruginosa mucoid strain. Infect Immun 45: 748-755.
40. Speert DP, Farmer SW, Campbell ME, Musser JM, Selander RK, et al. (1990) Conversion of Pseudomonas aeruginosa to the phenotype characteristic of strains from patients with cystic fibrosis. J Clin Microbiol 28: 188-194.

4

Inhibition of *Pseudomonas aeruginosa* Virulence

Characterization of the AprA-AprI interface and species selectivity

Bart W. Bardoel, Kok P.M. van Kessel, Jos. A.G. van Strijp, and Fin J. Milder

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands.

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Abstract

Pseudomonas aeruginosa secretes the virulence factor alkaline protease (AprA) to enhance its survival. AprA cleaves one of the key microbial recognition molecules, monomeric flagellin, and thereby diminishes Toll-like receptor 5 activation. In addition, AprA degrades host proteins such as complement proteins and cytokines. *P. aeruginosa* encodes a highly potent inhibitor of alkaline protease (AprI), that is solely located in the periplasm where it is presumed to protect periplasmic proteins against secreted AprA. We set out to study the enzyme-inhibitor interactions in more detail in order to provide a basis for future drug development.

Structural and mutational studies reveal that the conserved N-terminal residues of AprI occupy the protease active site and are essential for inhibitory activity. We constructed peptides mimicking the N-terminus of AprI, however these were incapable of inhibiting AprA-mediated flagellin cleavage. Furthermore, we expressed and purified AprI of *P. aeruginosa* and the homologous (37% sequence identity) AprI of *Pseudomonas syringae*, which, remarkably, show species specificity for their cognate protease. Exchange of the first five N-terminal residues between AprI of *P. syringae* and *P. aeruginosa* did not affect the observed specificity, whereas, exchange of only six residues located at the AprI surface that contacts the protease did abolish specificity. These findings are elementary steps towards the design of molecules derived from the natural inhibitor of the virulence factor AprA and their use in therapeutic applications in *Pseudomonas* and other Gram-negative infections.

Introduction

Pseudomonas species are common environmental Gram-negative bacteria that can cause infection in a wide variety of hosts [1]. In humans, *Pseudomonas aeruginosa* is an important source of both community-acquired and hospital-acquired infections [2]. *P. aeruginosa* may for instance cause ulcerative keratitis, otitis externa, skin and soft tissue infections, pneumonias, urinary tract infections, and infection of the bloodstream. Furthermore, *P. aeruginosa* is well known for its ability to form biofilms that colonize the airways, and thereby resists antibiotic treatment in cystic fibrosis patients [3].

The innate immune system plays an important role in detection and destruction of microorganisms. Recognition of highly conserved bacterial molecules is crucial to counteract bacterial invasion. The Toll-like receptor family recognizes a wide variety of such molecules and their activation triggers the production of inflammatory cytokines and attracts cells of the immune system to the site of infection. The building blocks of bacterial flagella, flagellin, is recognized by Toll-like receptor 5 (TLR5) [4,5]. Polymerized flagellin forms long filaments that are essential for bacterial motility and virulence. TLR5 recognizes a domain of flagellin that is required for filament assembly and motility [6]. Recognition of the highly conserved flagellin by the innate immune system enables efficient clearance of a wide range of microorganisms.

To minimize recognition and subsequent activation of the host immune system, bacteria secrete multiple inhibitory proteins and modify their own conserved molecules (e.g. flagellin) [7]. *P. aeruginosa* secretes virulence factors such as the proteases elastase and alkaline protease. Elastase degrades different types of collagens, which enhances bacterial invasion [8], and is also involved in the degradation of numerous components of the innate and adaptive immune system including surfactants [9], surface receptors [10] and cytokines [11]. Alkaline protease degrades the central complement protein C3 [12], IFN-gamma [11] and flagellin (Chapter 2). Alkaline protease degrades the monomeric form of flagellin, whereas the polymeric form, which is present in flagella and important for motility, is not degraded. Degraded flagellin is not recognized by TLR5 and emphasizes that the secreted protease AprA is an important virulence factor, involved in evasion of the immune system by *P. aeruginosa*.

Alkaline protease is a 50 kD zinc metalloprotease that is secreted by *P. aeruginosa*, via a type I secretion system, and belongs to the serralysin family [13]. Serralysins are wide spread virulence factors in Gram-negative bacteria including *Serratia* and *Erwinia* species [14]. The three genes that encode AprA its own secretion system are located directly upstream of the *aprA* gene. In addition, the gene located directly downstream of the *aprA* gene encodes a highly specific inhibitor of alkaline protease designated alkaline protease inhibitor (AprI). The exact biological function of this inhibitor remains unknown, since alkaline protease is a secreted protein and AprI is predicted to be a periplasmic protein. Putatively, AprI is part of a safety mechanism that provides protection of self-proteins for degradation by AprA prior to secretion. The apr gene cluster containing AprA, AprI and the genes that encode the secretion system are conserved among *Pseudomonas* species, *S. marcescens* and *E. chrysanthemi* [15].

AprA and AprI form a strong enzyme-inhibitor complex with a dissociation constant of 4 pM [16]. In the crystal structure of this bi-molecular complex AprI contacts AprA through its N-terminus (residues 1-5), the β-turn connecting β-strands s4 and s5, and β-strand s3. The

N-terminal trunk of AprI protrudes from the typical β-barrel structured protein and occupies the active-site cleft of AprA (Fig. 1A) [17]. Additionally, the first AprI residue, a serine, coordinates the catalytic zinc ion and, putatively, may contribute significantly to the high affinity of the inhibitor towards the protease. The importance of the N-terminus is further illustrated by the decreased affinity of AprI lacking the first 2-5 N-terminal residues, whereas no complex is formed upon deletion of the sixth residue as well [16]. The interactions between the β-barrel of the inhibitor and AprA may be crucial in the exact positioning of the extended N-terminal segment, such that the zinc is chelated properly and further insertion of the N-terminal segment and subsequent proteolysis is prevented.
As a very strong inhibitor of the important virulence factor AprA, the protease inhibitor AprI may provide a basis for the development of molecules derived from this natural inhibitor that facilitate treatment of *P. aeruginosa* related diseases. This concept was recently demonstrated for *Pseudomonas* elastase [18]. Several related Gram-negative bacteria also express AprA, and therefore therapeutic potential could well go beyond *Pseudomonas* alone. As such it is evident that understanding the enzyme-inhibitor interactions at the molecular level and the exact species specificity in more detail is pivotal for future drug research. We set out to identify the amino acids involved using protein mutagenesis and functional experiments. Multiple synthetic peptides, corresponding with the AprI N-terminus, were tested for their inhibitory capacity. Furthermore, AprI proteins were produced with mutations in their N-terminal region (amino acid exchange and deletions) and AprA interface region. These AprI mutants were biochemically and functionally characterized.

Materials and Methods

AprI isolation

WT AprI from *P. aeruginosa* ($AprI^{PA}$) and *P. aeruginosa* AprI with *P. syringae* interface (ASI) (Fig. 3B), were isolated under denaturing conditions. The gene *aprI* and ASI without signal sequence of *P. aeruginosa* strain PAO1 with an *Xba*1 restriction site, 6x His-tag and enterokinase cleavage fused to the 5'-end and a 3' *Eco*R1 restriction site was synthesized by BaseClear (Leiden, The Netherlands). The *aprI* construct was ligated into a pRSETB vector and transformed into *E. coli* Top10F' cells, according to manufacturer's protocol (Invitrogen). Protein expression was performed in *E. coli* BL21 grown in LB medium at 37°C and induced with 1 mM isopropyl-1-thio-b-D-galactopyranoside (IPTG, Roche). After 4 hours of induction, bacteria were harvested and pellets were lysed with 6 M guanidine + 0.5 M NaCl + 20 mM sodium phosphate pH 7.8. Proteins were purified under denaturing conditions using a Hi trap chelating column loaded with $Ni2^{+}$ (GE Healthcare) equilibrated with 8 M ureum + 0.5 M NaCl + 20 mM sodium phosphate pH 7 and eluted with 0.5 M imidazole + 0.5 M NaCl + 8 M ureum + 20 mM sodium phosphate pH 8.5. Purified denatured protein was diluted 10-fold in PBS and concentrated on a His trap column. Purity of renatured AprI was assessed by SDS-PAGE and the protein was dialyzed against PBS.
WT AprI from *P. syringae* ($AprI^{PS}$), *P. aeruginosa* AprI with *P. syringae* N-terminus (ASN), *P. syringae* AprI with *P. aeruginosa* N-terminus (SAN), *P. syringae* AprI with *P. aeruginosa* interface (SAI1-4), and *P. aeruginosa* AprI without the first five residues (A-5N) (Fig. 2B and 3B) were isolated under native conditions. All genes were synthesized (Geneart, Invitrogen) except for AprI from *P. syringae* DC3000 (PSPTO-3331), which was cloned by overlap

extension PCR [19]. *P. syrinage* AprI was amplified from chromosomal *P. syringae* DC3000 DNA using the primers $AprI^{PS}$: 5'-GGA TCT GTA CGA CGA TGA CGA TAA GAT GAG TTT AAA ACT GCC CAA C-3' (forward) and 5'- GAT CGA ATT CCT TAA TCG GCG AAC CGT CCA A-3' (reverse) and cloned directly downstream of the enterokinase cleavage site of pRSETB. For ASN and SAN *E. coli* strain BL21 (Novagen) was used as expression strain and for the other proteins Rosetta gami (Invitrogen). Protein isolation was performed under native conditions using CellyticB (Sigma) supplemented with 10 µg/ml DNAse/RNAse (Roche) and EDTA free protease inhibitor cocktail (Roche). Cell lysate was applied on a His trap FF column after centrifugation and filtration (0.45 µm filter). Column was washed with 0.5 M NaCl + 20 mM sodium phosphate pH 7 + 20 mM imidazole and eluted with the same buffer containing 0.5 M imidazole.

For enterokinase cleavage, His-tagged proteins were incubated with 0.3-3 U enterokinase per mg protein (Invitrogen) in 50 mM Tris pH 8 + 1 mM $CaCl_2$ for 2 hours at 37°C. After cleavage sample was loaded on a His trap FF (GE Healthcare) column equilibrated with PBS and the flow through was collected and analyzed for purity by SDS-PAGE and Coomassie staining (Instant Blue, Expedeon protein solutions). Protein concentration was quantified by measuring UV absorbance at 280 nm and with the BiCinchoninic Acid protein assay (Pierce).

AprA isolation

The gene *aprA* of *P. aeruginosa* was amplified from genomic PAO1 DNA using a forward and reverse primer with incorporated 5'BamH1 and 3'Not1 restriction sites, respectively. The PCR product encodes for the AprA protein without the nine-residue propeptide. For *P. syringae aprA*, the complete gene was amplified with the same restriction sites using *P. syringae pv. tomato* DC3000 DNA as template. PCR products were ligated in a modified pET302 vector (Invitrogen) encoding an N-terminal hexa-histidine followed by a tobacco etch virus (TEV) protease site and transformed into Top10F'cells (Invitrogen). After sequence verification, the constructs were transformed into the *E. coli* Rosetta Gami (DE3) pLysS (Novagen). Bacteria were cultured in LB medium at 37°C and protein expression was induced by adding 1 mM IPTG. After 4 hours induction, bacteria were pelleted and lysed by 6 M guanidine + 0.5 M NaCL + 20 mM sodium phosphate pH 7.8. His-tagged AprA was purified under denaturing conditions using a Hi trap chelating column as described for AprI. Denatured AprA was diluted 50 times in 0.8 M L-arganine + 50 mM Tris-HCl pH 9 + 1 mM $CaCl_2$ at 4°C overnight. After renaturation, AprA was concentrated on a Amicon 30 kD spin column (Millipore) and washed two times with 50 mM HEPES pH 7.8 + 1 mM $CaCl_2$ + 0.1 mM $ZnCl_2$. Purity was analyzed with SDS-PAGE and Coomassie staining. AprA was dialyzed against PBS, and stored at -20°C.

AprI peptides

Peptides mimicking the N-terminal part of *P. aeruginosa* AprI were synthesized by Genscript (Piscataway, New Jersey, USA). In total 4 peptides were prepared, two peptides comprising the first 10 and 15 amino acids and two similar peptides with the I4W mutation that results in AprI that binds AprA with a higher affinity [20] compared to WT AprI. Peptides were dissolved in DMSO at a concentration of 1 mM and stored at -20°C.

Circular dichroism

Prior to measuring the far-UV circular dichroism (CD) spectra (190-250 nm) the proteins were dialyzed against 25 mM NaCl + 20 mM sodium phosphate pH 7. The CD spectra of the protein samples were recorded at an AprI concentration of 400 µg/ml at room temperature in a quartz cell (path length 0.05 cm) using a dual-beam DSM 1000 CD spectrophotometer (On-line Instruments System Borgart, GA, USA). Three scans of each sample were averaged.

Flagellin cleavage

Recombinant *P. aeruginosa* flagellin at 250 µg/ml was incubated with AprA and AprI variants in PBS for 1 h at 37°C. Subsequently, sample buffer was added and samples were analyzed on a 15 % SDS-PAGE gel and stained with Coomassie.

AprA-AprI complex formation

AprA (200 µg/ml) and AprI variants (100 µg/ml) were premixed and applied onto a Superdex 75 column (GE Healthcare) equilibrated with 50 mM Tris pH 7.8 + 100 mM NaCl and 1 mM $CaCl_2$ using an AKTA FPLC (GE healthcare). OD 280 nm was measured to determine the retention times of both isolated AprA and AprI and the AprA-AprI complex.

Substrate cleavage

The substrate Ac-Pro-Leu-Gly-[2-mercapto-4-methyl-pentanoyl]-Leu-Gly ethyl ester (Bachem) was used to measure AprA activity [16,20,21]. Substrate hydrolysis was determined by using 500 µM of the thiol indicator DTDP (Sigma) dissolved in 50 mM MOPS pH 7 + 5 mM $CaCl_2$ + 100 mM NaCl. AprA was preincubated with buffer or AprI variants and added to this assay buffer. Substrate cleavage was monitored in a quartz cuvette (1 cm optical pathway) at 324 nm using a Genesys 10 UV spectrophotometer (Thermo Spectronic, Rochester, NY). For dissociation experiments, 650 nM AprA was incubated with 500 nM AprI variants for at least one hour in assay buffer at room temperature. To measure dissociation, the protease-inhibitor complex was diluted 100 times in assay buffer containing 2.4 M NaCl and absorbance was measured at 324 nm.

Accession numbers

Swiss-prot accession numbers: *P. aeruginosa* AprA (Q03023), AprI (Q03026), flagellin type B (P72151) from *P. aeruginosa*, *P. syringae* AprA (Q87ZU2) and *P. syringae* AprI (Q87ZU3). PDB accession number: *P. aeruginosa* AprA-AprI complex (1JIW).

Results

N-terminal AprI peptides show no inhibitory activity

P. aeruginosa AprI with a truncated or mutated N-terminus showed impaired inhibitory activity, indicating that the first five amino acids are essential for a fully functional protein [16]. Furthermore, AprI with the fourth residue mutated to a tryptophan (I4W) expressed an even higher affinity for AprA compared to wild-type AprI [20]. So far, all mutagenesis studies were performed using AprI with altered N-termini. The full-length protein is unsuitable for future drug development, because of inherit immunogenicity. We set out to determine

whether the N-terminal trunk by itself does suffice to functionally inhibit AprA-mediated cleavage of flagellin. Two peptides identical to the important first 10 and 15 AprI residues (Fig. 1B) and two homologous peptides with the I4W substitution were examined.
The full-length protein AprI inhibited AprA-mediated degradation of flagellin efficiently (Fig. 1C). Inhibition of AprA was not observed upon addition of the synthetic peptides. The cleavage of flagellin in the presence of the N-terminus mimicking peptides is, based on SDS-PAGE analysis, identical to incubation with solely the protease. These findings indicate that the AprI N-terminus by itself does not inhibit the protease activity of AprA.

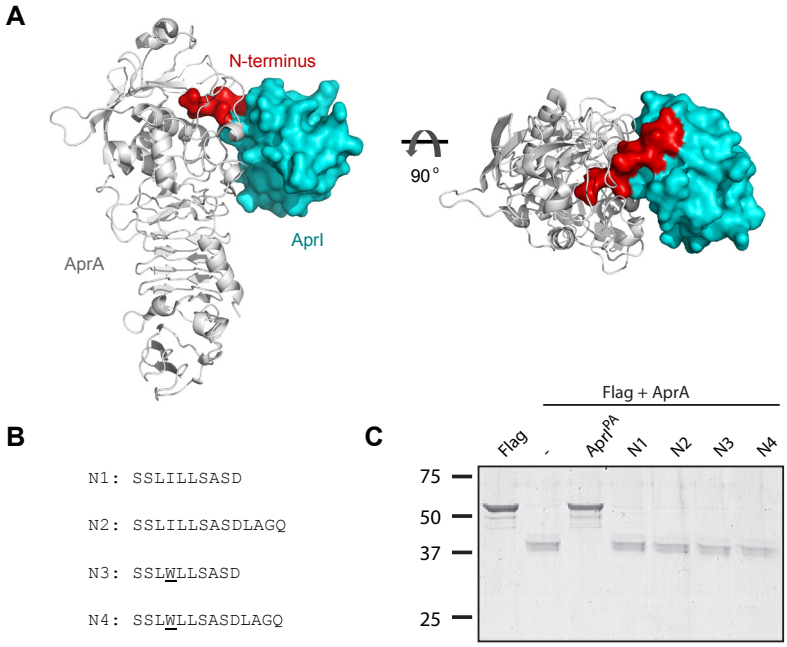

Figure 1. Synthetic peptides homologous to the AprI N-terminus do not inhibit AprA activity
(A) Crystal structure of AprA (white) shown in ribbon representation in complex with AprI (light blue) shown in surface representation. The AprI N-terminus (red) occupies the active-site cleft of AprA. (B) The four synthetic peptides: two identical to the first 10 and 15 residues of AprI^{PA}, N1 and N2 respectively, and two with the I4W (N3 and N4). (C) Flagellin (250 µg/ml) cleavage by AprA^{PA} (1 µg/ml) in presence of wild-type AprI^{PA} (10 µg/ml) or AprI peptides (10 µM) for 1 hour at 37°C and analyzed by SDS-PAGE and Coomassie staining.

Species specificity

To further investigate the high affinity AprA-AprI interaction the homologous proteins of plant pathogen *P. syringae* were cloned, expressed, and functionally characterized. These *P. syringae* proteins have an amino acid identity of 63% and 37% with *P. aeruginosa* AprA (AprA^{PA}) and AprI, respectively.
As expected proteases of both species degraded flagellin, however with different degradation products (Fig. 2A); a single product (± 37 kD) for AprA of *P. aeruginosa* and a double product (± 40 and 43 kD) for AprA of *P. syringae*. The inhibitor was inactive across species; no inhibition of *P. aeruginosa* AprA by *P. syringae* AprI was observed and vice versa. AprA-AprI complex formation can be monitored by size-exclusion chromatography. Based on the peak shifts of the individual components it was evident that AprA-AprI complexes

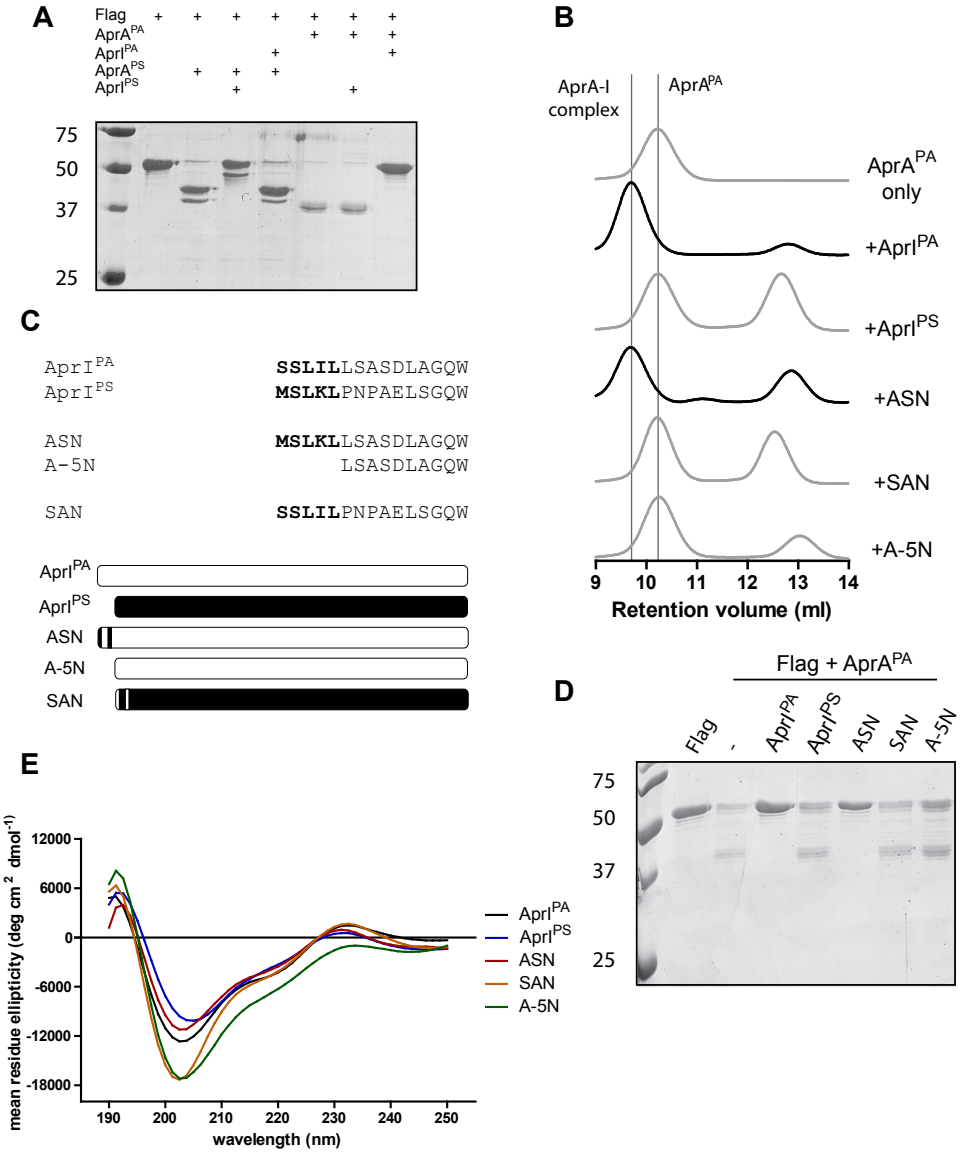

Figure 2. Species specificity not determined by AprI N-terminus
(A) *P. aeruginosa and P. syringae* AprA-mediated (3 µg/ml and 30 µg/ml, respectively) flagellin cleavage (250 µg/ml) in the presence or absence of 30 µg/ml *P. aeruginosa or P. syringae* AprI for 1 hour at 37 °C and analyzed by SDS-PAGE and Coomassie staining. (B) Analysis of AprA^{PA}-AprI (200 and 100 µg/ml, respectively) complex formation by size-exclusion chromatography. Based on the absorbance at 280 nm the retention volume of enzyme-inhibitor, enzyme, and inhibitor are 9.7, 10.2, and 12.5-13 ml, respectively. (C) Upper panel, the amino acid sequences of the N-termini (bold) of wild-type AprI^{PA}, AprI^{PS}, AprI mutants ASN and SAN, and AprI^{PA} lacking the first 5 N-terminal residues (A-5N). Lower panel, schematic representation of the AprI mutants; in white residues of *P. aeruginosa* and in black residues of *P. syringae*. (D) Functional analysis of wild-type AprI from *P. aeruginosa* and *P. syringae*, and the 3 AprI mutants; ASN, SAN, and A-5N. Flagellin (250 µg/ml) was incubated with AprA^{PA} (3 µg/ml) and the AprI mutants (10 µg/ml) for 1 hour at 37°C. Flagellin degradation was analyzed by SDS-PAGE and Coomassie staining. (E) Secondary structure analysis of the AprI mutants by circular dichroism.

were formed within one species, whereas no complexes were formed across species (Fig. 2B). These data indicate species specificity of AprA and AprI that directly correlates to the ability to form enzyme-inhibitor complexes.

The AprI N-terminus does not determine species specificity

The N-termini of *P. syringae* ($AprI^{PS}$) and *P. aeruginosa* AprI ($AprI^{PA}$) differ at positions 1 and 4 (Fig. 2C). The significance of the N-terminal residues for the biological function of $AprI^{PA}$ is demonstrated by the loss of function of the A-5N mutant; truncated AprI did not bind to AprA and did not inhibit the protease activity (Fig. 2B and 2D).

To examine if the observed species selectivity resides in this functionally important part of the inhibitor, two chimeric AprI proteins were cloned and expressed in which the N-termini of both species were exchanged (Fig. 2C). Both proteins were tested for their capacity to inhibit $AprA^{PA}$. No difference was observed in activity for wild-type $AprI^{PA}$ and chimeric AprI (ASN) in the functional assay (Fig. 2D). Both proteins inhibited AprA-mediated flagellin cleavage. Furthermore, *P. syringae* AprI with the *P. aeruginosa* N-terminus (SAN) did, similar to $AprI^{PS}$, not inhibit $AprA^{PA}$-mediated flagellin degradation. As expected, the capacity of complex formation was directly related to the observed inhibitory activity of chimeric AprI (Fig. 2B).

To ensure that the results presented above are not ascribed to improper protein folding, the content of the secondary-structure elements of wild-type and the constructed chimeric AprI proteins was determined by recording their far-UV circular dichroism spectra (Fig. 2E). The similarity in overall shapes of the spectra indicates no major differences in the overall structure of all AprI proteins. The relative content of α-helices, β-strands, and unstructured element (loops) was estimated by the online program CONTINLL to be 5.9 ± 2%, 35.1 ± 4.2%, and 59 ± 3.2%, respectively [22].

To summarize, these data indicate the N-terminus, although its presence is a prerequisite for complex formation and functional inhibition of AprA, does not account for the observed species specificity and at least a two amino acid exchange at position 1 and 4 is tolerated.

Species specificity abolished by a six-residue exchange within the inhibitor main body

AprI interacts with AprA though multiple contact sites other than the N-terminus. These enzyme-inhibitor contacts are located on one side of the spherical shaped AprI β-barrel. The crystal structure of the AprA-AprI complex revealed at least 16 residues of the inhibitor that interact with the protease. Six of these are identical in AprI of both *P. aeruginosa* and *P. syringae* (Fig. 3A and 3B). Multiple chimeric *P. syringae* AprI molecules were functionally characterized to further investigate the role of the 10 unique interface residues in protein-protein interaction.

Remarkably, *P. syringae* AprI in which the 10 unique residues at the interface region were mutated to homologous residues of *P. aeruginosa* AprI (SAI1) (Fig. 3B), bound to and functionally inhibited $AprA^{PA}$ (Fig. 3C and 3D). The *P. aeruginosa* AprI equivalent of SAI1 AprI (ASI, *P. aeruginosa* AprI with the *P. syringae* AprI interface) did not bind to $AprA^{PA}$ and did not inhibit its proteolytic activity (Fig. 3B-3D). The second *P. syringae* AprI interface mutant (SAI2) comprises six out of the ten selected residues that contribute most to the buried-surface area of the protein-protein interface (Fig. 3B). This chimeric protein showed binding and functional activity identical to $AprA^{PA}$ and a similar the gain of function as observed for mutant SAI1. The six residues of SAI2 spatially cluster in two groups of three residues. In

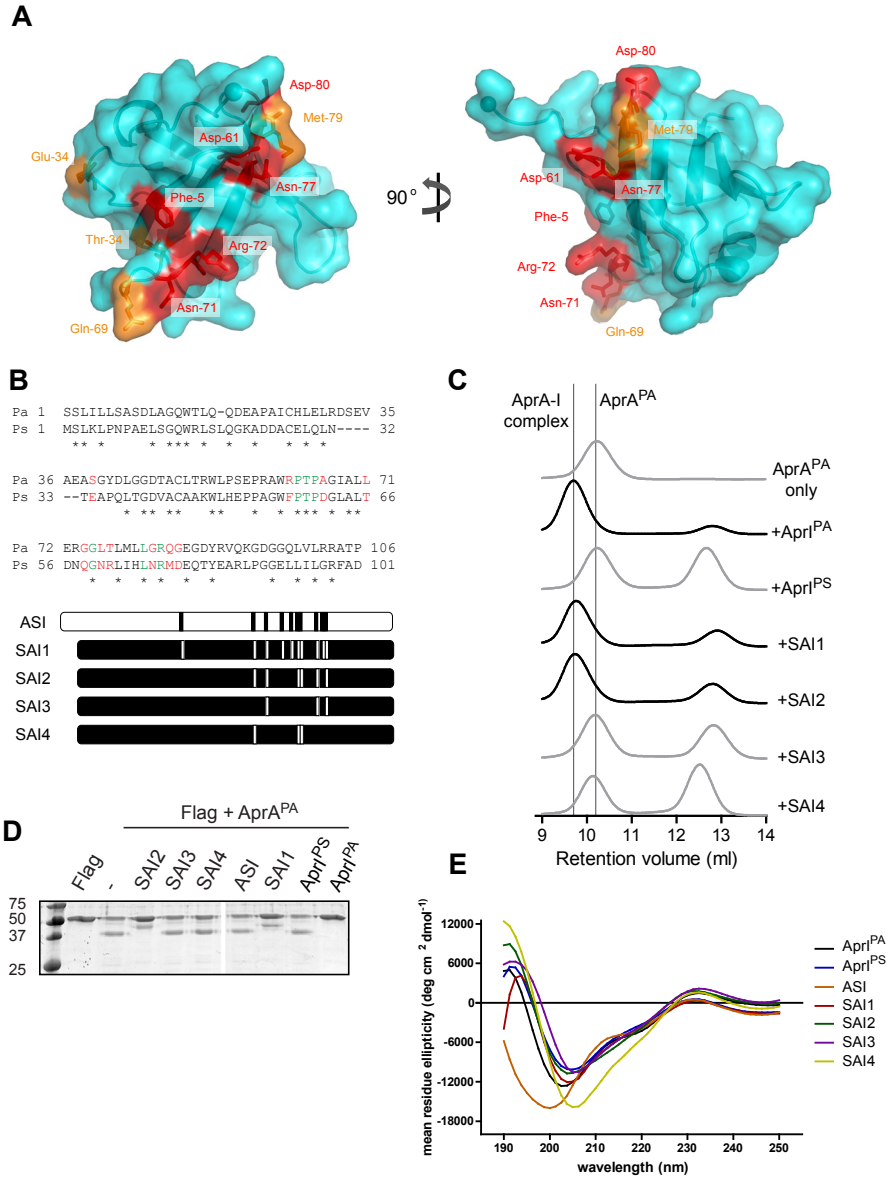

Figure 3. AprA-AprI interface and species specificity
(A) Shown in surface representation is a SWISS Model generated structure of AprI^{PS}; the ten non-homologous AprI interface residues are highlighted in orange (non-crucial) and red (crucial). (B) Upper panel, amino acid sequence of *P. aeruginosa and P. syringae* AprI; homologous interface residues are highlighted in green, non-homologous residues are highlighted. Lower panel, schematic overview of the interface mutants; in white residues of *P. aeruginosa* and in black residues of *P. syringae*. (C) AprA^{PA}-mediated (3 µg/ml) cleavage of flagellin (250 µg/ml) in the presence of the AprI interface mutants. After one hour at 37°C cleavage was analyzed by SDS-PAGE and Coomassie staining. (D) Analysis of AprA^{PA}-AprI (200 and 100 µg/ml, respectively) complex formation by size-exclusion chromatography, comparison of wild-type AprI and AprI interface mutants. Based on the absorbance at 280 nm the retention volume of enzyme-inhibitor, enzyme, and inhibitor are 9.7, 10.2, and 12.5-13 ml, respectively. (E) Secondary structure analysis of AprI interface mutants by circular dichroism.

order to further pinpoint the crucial interactions, two additional interface mutants were prepared (SAI3 and SAI4, Fig. 3B). However, both these three-residue *P. syringae* AprI mutants displayed no binding to $AprA^{PA}$ and no functional activity (Fig. 3C and 3D). Similar to the N-terminal AprI mutant proteins folding was verified by CD spectroscopy (Fig. 3E). Except for the ASI chimera the overall shapes of the CD-spectra from the interface mutants were very similar.

Biochemical analysis of AprI mutants

Analysis of AprA-mediated flagellin degradation is an appropriate readout for biological activity, however minor differences in activity are difficult to quantify. To circumvent this drawback, activity was determined by measuring the conversion of a synthetic substrate by AprA and the inhibition thereof by WT and mutant AprI. A dose-dependent inhibition of substrate conversion was observed for *P. aeruginosa* AprI with an IC50 at an equimolar AprA-AprI concentration (Fig. 4A). All mutants that did not functionally inhibit flagellin degradation were incapable to reduce AprA mediated substrate conversion (Fig. 4B). In addition, inhibition of synthetic substrate conversion was observed by the mutants SAI1, SAI2 and ASN, that inhibited $AprA^{PA}$-mediated flagellin degradation.

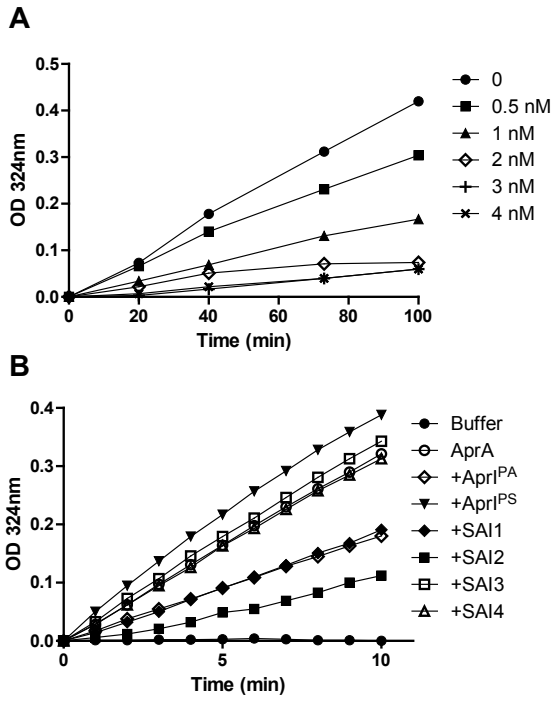

Figure 4. AprA-mediated substrate conversion inhibited by AprI interface mutants
(A) conversion of synthetic substrate Ac-Pro-Leu-Gly-[2-mercapto-4-methyl-pentanoyl]-Leu-Gly ethyl ester (300 µM) by $AprA^{PA}$ (1 nM) in presence of $AprI^{PA}$ (0, 0.5, 1, 2, 3, and 4 nm). Reaction was performed in assay buffer. Absorbance at 324 nm was measured every 20 min. (B) $AprA^{PA}$ (500 nM) and AprI interface mutants (650 nM) were incubated for 1 hour at room temperature in assay buffer without DTDP and substrate. The enzyme-inhibitor mixture was subsequently diluted 100 times in assay buffer containing 2.4 M NaCl, 500 µM DTDP and 100 µM synthetic substrate. Absorbance at 324 nm was measured every minute.

Discussion

Neutralization of pathogenic virulence factors is crucial in the treatment of immune-compromised patients that suffer from severe *Pseudomonas* related infections [18]. The secreted virulence factor alkaline protease (AprA) is effectively inhibited by alkaline protease inhibitor (AprI), that is predicted to be localized in the periplasmic space of *P. aeruginosa*. Molecules derived from this natural inhibitor may in part restore the host immune responses by recovering proper flagellin-mediated TLR5 signaling. The crystal structure of the inhibitor of *Erwinia chrysanthemi* bound to the metalloprotease of *Serratia marcescens* indicates the inhibitors from the serralysin family are active across strain and species [15]. In this study, we show this is not the case for the serralysin inhibitors of *P. aeruginosa* and *P. syringae* and we analyzed the role of the functionally important N-terminal residues [20] and the contact sites of the AprI main body with AprA in this process. Since AprI mediated inhibition of AprA may facilitate future therapeutic applications that neutralize this wide spread virulence factor, the aspects of species specific interactions is of crucial importance. Synthetic peptides homologous to the N-terminus of AprI do not display inhibitory activity; no difference in AprA mediated cleavage of flagellin was observed in the presence of these peptides. Putatively the peptides may not bind correctly to the protease. As was proposed by Hege *et al.* [17], proper alignment of the N-terminus of AprI may be dependent on the presence of the protein main body that prevents the N-terminus from being pulled further in the active site cleft as a true substrate. Alternatively the peptide may not, or only very weakly bind in absence of this domain. As such the binding of the true substrate, monomeric flagellin, is not affected.

AprI of *P. syringae* does not inhibit *P. aeruginosa* AprA and vice versa. Although the end products differ, AprA of both strains degrade flagellin. This is probably due to a slight difference in protease activity or protein concentration, because flagellin cleavage using lower $AprA^{PA}$ concentrations results in end products similar in size as observed for $AprA^{PS}$. Sequence conservation of AprA between the strains is relatively high when compared with its cognate inhibitor AprI, 64% and 38%, respectively. *P. aeruginosa* AprI shares a similar sequence conservation with the AprI homologs of *S. marcescens* and *E. chrysanthemi* (37-39%), whereas the sequence conservation of AprA is slightly lower (52-54%), compared to *P. syringae*. In the crystal structure of the *S. marcescens* AprA in complex with *E. chrysanthemi* AprI [15], the N-terminus of the inhibitor is tightly embedded in the protease substrate-binding groove and accounts for most protein-protein contacts. A similar conformation was observed for the enzyme-inhibitor complex of *P. aeruginosa* [20]. Replacement of the *P. aeruginosa* AprI N-terminus by homologous residues of *P. syringae* AprI does not affect the protein function; inhibition of AprA mediated flagellin cleavage and complex formation with AprA is identical to WT AprI. The residues involved are Ser-1 and Ile-4; residues 2, 3, and 5 are identical in both species. The amino and carbonyl groups of the N-terminal serine residue chelate the catalytic zinc ion [17] and likely account for the high affinity of the enzyme-inhibitor complex. In addition, the side-chain hydroxyl group contributes by hydrogen bonding with the hydroxyl oxygen of AprA Tyr-216. In the ASN mutant the serine is replaced by a methionine. Putatively, the extended substrate-binding groove facilitates the larger methionine side chain and thereby minimizes steric hindrance. Both main-chain interactions with the zinc ion are likely unaffected, however the hydrogen bonding of the serine side-chain is lost. This hydrogen bond is not crucial for AprA-AprI complex formation

as was shown by a study of Feltzer et al. [20] in which mutation of the N-terminal serine to a glycine yielded a complex with even a lower Kd value. The mutation at position four results in the exchange of a hydrophobic isoleucine to the positively charged lysine. The amino acid at this position is not conserved along the known serralysin inhibitors and a mutation to an arginine, similar in charge and size to lysine, affects the AprI-AprA interaction only minor [20]. These data indicate the N-terminus is not the main determinant in the observed species specificity of AprI mediated inhibition of AprA in between *P. aeruginosa* and *P. syringae*.

The crystal structures of *P. aeruginosa* AprA-AprI [17] and serralysin of *S. marcescens* in complex with the serralysin inhibitor of *E. chrysanthemi* [15] demonstrated that the β-turn connecting β-strands s4 and s5, and β-strand s3 of the inhibitor interact with the protease. The exchange of six amino acids located on the enzyme-inhibitor interface abolished the observed species selectivity of AprI. Based on the crystal structure of *P. aeruginosa* AprI, a model of *P. syringae* AprI was generated by the protein structure homology modelling server SWISS-MODEL [23]. Superposition of both molecules in the *P. aeruginosa* enzyme-inhibitor complex indicated two putative mismatches; (i) Asp-61, located in the β-turn connecting strands s3 and s4, and (ii) Arg-72, located in the β-turn connecting strands s4 and s5. The hydrophilic Asp-61, an alanine in *P. aeruginosa* AprI, is directed towards a hydrophobic pocket formed by AprA residues Tyr-216 and Trp-217 and AprI residue Leu-3. Accommodation of a charged residue in this site may be unfavorable and putatively interferes with correct stabilization of the conserved Leu-3, part of crucial N-terminus, by Apr-A residues Tyr-216, Trp-217, and Tyr-169. In the model of *P. syringe* AprI Arg-72, a threonine in *P. aeruginosa* AprI, protrudes from the protein surface and clashes with AprA residues Gln-220, Gln-224, and Asp-225. Furthermore, Asp-61 and Arg-72 account for three out of the five hydrogen bonds in the enzyme-inhibitor interface (exclusive the N-terminal interactions). Putatively, rearrangement of the local conformation through steric hindrance may perturb these interactions that significantly contribute to the AprA-AprI affinity.

In this study, we show the AprI N-terminus by itself does not suffice to functionally inhibit AprA-mediated flagellin cleavage. Furthermore, species specificity is observed between *P. aeruginosa* and *P. syringae*. Surprisingly, this effect is abolished upon mutation of only six *P. syringae* AprI interface residues into homologous $AprI^{PA}$ residues. The data presented above provide new insight in the enzyme-inhibitor interactions and may prove useful in the development of an AprI-derived molecule that efficiently neutralizes the virulence factor AprA. This modified AprA inhibitor is suitable for therapeutic applications in diseases caused by *Pseudomonas* and even other related Gram-negative species.

Acknowledgements

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References

1. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, et al. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. Science 268: 1899-1902.
2. Lyczak JB, Cannon CL, Pier GB (2000) Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. Microbes Infect 2: 1051-1060.
3. Jensen PO, Givskov M, Bjarnsholt T, Moser C (2010) The immune system vs. Pseudomonas aeruginosa biofilms. FEMS Immunol Med Microbiol 59: 292-305.
4. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, et al. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410: 1099-1103.
5. Feuillet V, Medjane S, Mondor I, Demaria O, Pagni PP, et al. (2006) Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria. ProcNatlAcadSciUSA 103: 12487-12492.
6. Smith KD, Andersen-Nissen E, Hayashi F, Strobe K, Bergman MA, et al. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol 4: 1247-1253.
7. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, et al. (2005) Evasion of Toll-like receptor 5 by flagellated bacteria. ProcNatlAcadSciUSA 102: 9247-9252.
8. Matsumoto K (2004) Role of bacterial proteases in pseudomonal and serratial keratitis. Biol Chem 385: 1007-1016.
9. Alcorn JF, Wright JR (2004) Degradation of pulmonary surfactant protein D by Pseudomonas aeruginosa elastase abrogates innate immune function. J Biol Chem 279: 30871-30879.
10. Leduc D, Beaufort N, de Bentzmann S, Rousselle JC, Namane A, et al. (2007) The Pseudomonas aeruginosa LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain-specific endoproteolysis. Infect Immun 75: 3848-3858.
11. Parmely M, Gale A, Clabaugh M, Horvat R, Zhou WW (1990) Proteolytic inactivation of cytokines by Pseudomonas aeruginosa. Infect Immun 58: 3009-3014.
12. Hong YQ, Ghebrehiwet B (1992) Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol 62: 133-138.
13. Tommassen J, Filloux A, Bally M, Murgier M, Lazdunski A (1992) Protein secretion in Pseudomonas aeruginosa. FEMS Microbiol Rev 9: 73-90.
14. Stocker W, Grams F, Baumann U, Reinemer P, Gomis-Ruth FX, et al. (1995) The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci 4: 823-840.
15. Baumann U, Bauer M, Letoffe S, Delepelaire P, Wandersman C (1995) Crystal structure of a complex between Serratia marcescens metallo-protease and an inhibitor from Erwinia chrysanthemi. J Mol Biol 248: 653-661.
16. Feltzer RE, Gray RD, Dean WL, Pierce WM, Jr. (2000) Alkaline proteinase inhibitor of Pseudomonas aeruginosa. Interaction of native and N-terminally truncated inhibitor proteins with Pseudomonas metalloproteinases. J Biol Chem 275: 21002-21009.
17. Hege T, Feltzer RE, Gray RD, Baumann U (2001) Crystal structure of a complex between Pseudomonas aeruginosa alkaline protease and its cognate inhibitor: inhibition by a zinc-NH2 coordinative bond. J Biol Chem 276: 35087-35092.
18. Cathcart GR, Quinn D, Greer B, Harriott P, Lynas JF, et al. (2011) Novel Inhibitors of the Pseudomonas aeruginosa Virulence Factor LasB: a Potential Therapeutic Approach for the Attenuation of Virulence Mechanisms in Pseudomonal Infection. Antimicrob Agents Chemother 55: 2670-2678.
19. Jongerius I, Kohl J, Pandey MK, Ruyken M, van Kessel KP, et al. (2007) Staphylococcal complement evasion by various convertase-blocking molecules. J Exp Med 204: 2461-2471.
20. Feltzer RE, Trent JO, Gray RD (2003) Alkaline proteinase inhibitor of Pseudomonas aeruginosa: a mutational and

molecular dynamics study of the role of N-terminal residues in the inhibition of Pseudomonas alkaline proteinase. J Biol Chem 278: 25952-25957.

21. Weingarten H, Feder J (1986) Cleavage site specificity of vertebrate collagenases. Biochem Biophys Res Commun 139: 1184-1187.

22. Whitmore L, Wallace BA (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res 32: W668-673.

23. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195-201.

5

Identification of an Immunomodulating Metalloprotease of *Pseudomonas aeruginosa* (IMPa)

Bart W. Bardoel, Dennis Hartsink, Mignon M. Vughs, Carla J.C. de Haas, Jos A.G. van Strijp, and Kok P.M. van Kessel

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands.

Manuscript submitted

Abstract

Phagocytosis by neutrophils is the essential step in fighting *Pseudomonas* infections. The first step in neutrophil recruitment to the site infection is the interaction of P-selectin (on endothelial cells) with P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils. *Pseudomonas aeruginosa* secretes various proteases that degrade proteins that are essential for host defense, such as elastase and alkaline protease. Here we identify PA0572 of *P. aeruginosa* as an inhibitor of PSGL-1 and named this secreted hypothetical protease immunomodulating metalloprotease of *Pseudomonas aeruginosa* or IMPa. Proteolytic activity was confirmed by cleavage of recombinant and cell-surface expressed PSGL-1. Functional inhibition was demonstrated by impaired PSGL-1-mediated rolling of IMPa-treated neutrophils under flow conditions. Next to PSGL-1, IMPa targets CD43 and CD44 that are also involved in leukocyte homing. These data indicate that IMPa prevents neutrophil extravasation, and thereby protects *P. aeruginosa* from neutrophil attack.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is commonly found in soil and water. In humans, it acts as an opportunistic pathogen causing severe chronic infections in cystic fibrosis patients [1]. *P. aeruginosa* colonizes the cystic fibrosis lung where it forms biofilms that are insensitive to antimicrobial treatment [2]. In healthy hosts, the innate immune system very rapidly and efficiently kills invading micro-organisms. Gram-negative bacteria are lysed by complement via the membrane attack complex, however many *P. aeruginosa* strains are insensitive to complement-mediated killing [3]. Furthermore, this bacterium secretes several proteins that contribute to virulence and assist to circumvent different branches of the innate immune system [4]. The proteases elastase and alkaline protease of *P. aeruginosa* degrade components of the complement system [5], and immunoglobulins [6]. Expression of both proteases as well as other pseudomonal proteins are regulated via the transcriptional regulatory protein LasR that belongs to the quorum sensing system *las* [7,8]. This system contributes to *P. aeruginosa* virulence in vivo [9].

Production of pro-inflammatory cytokines and chemokines after recognition of conserved bacterial structures by the innate immune system attracts leukocytes to the site of infection. These inflammatory mediators induce the expression of E- and P-selectin [10]. P-selectin glycoprotein ligand-1 (PSGL-1) is the major ligand for P-selectin [11,12], however PSGL-1 also interacts with E-selectin [13]. Transient interactions between PSGL-1 and these two selectins initiate rolling of leukocytes over the endothelium, the first step in recruitment of leukocytes to the site of infection. PSGL-1 is expressed on most leukocytes including lymphocytes, monocytes and neutrophils. It is a heavily glycosylated transmembrane protein of 120 kD and expressed as a disulfide-linked homodimer on cells. PSGL-1 contains three N-linked glycans, sialylated and fucosylated O-linked glycans, which often terminate with a sialyl Lewis X oligosaccharide [14]. Proper glycosylation of PSGL-1 is required for interaction with P-selectin [15].

The glycoprotein CD44 expressed on leukocytes binds E-selectin and can cooperate with PSGL-1 to initiate rolling of neutrophils over the endothelium [16]. The extracellular matrix component hyaluronan is another ligand of this type I transmembrane cell-surface receptor [17]. Cell adhesion and migration by CD44 play a role in different biological processes like lymphocyte activation and tumor-cell migration. In addition to CD44, the heavily glycosylated leukosialin receptor (CD43) expressed on various leukocytes is also involved in cell adhesion [18]. The O-glycan structures on CD43 differ between cell types, lymphocytes contain a low and neutrophils a high molecular weight O-glycan structure. Some studies [19], but not others [20], suggest that CD43 also binds to E-selectin on activated T-cells. CD43, CD44 and PSGL-1 all contain mucin-type O-glycans, which are essential for the interaction with E-selectin [21].

Bacteria exploit several strategies to interfere with various components of the innate immune system [22]. The secreted protein staphylococcal superantigen-like 5 (SSL5) of *Staphylococcus aureus* binds to PSGL-1 in a sialyl Lewis X dependent way [23] and to other proteins that contain this carbohydrate structure [24]. SSL5 inhibits rolling of neutrophils on activated human umbilical vein endothelial cells. In the present study, we screened the secretome of *P. aeruginosa* for the presence of proteins that interfere with the P-selectin/PSGL-1 interaction. Prevention of anti-PSGL-1 monoclonal antibody binding to leukocytes treated with concentrated *P. aeruginosa* supernatant confirmed the existence of a PSGL-1

inhibitor. Fractionation of the supernatant followed by mass spectrometry analysis revealed that the hypothetical metalloprotease PA0572 exerts proteolytic activity for PSGL-1 and three other leukocyte cell-surface receptors.

Materials and methods

IMPa isolation

Pseudomonas aeruginosa (PAO1) was cultured overnight at 37°C in Luria broth (LB). Supernatant was collected by centrifugation and proteins were precipitated with ammonium sulfate (60% saturation) overnight at 4°C. Precipitated proteins were pelleted, dissolved in distilled water and dialyzed against 20 mM sodium phosphate pH 7. Sample was loaded on a DEAE column (GE Healthcare), washed with phosphate buffer and eluted with the same buffer + 1 M NaCl. Active fractions in the antibody competition assay were concentrated using a 30 kD cut-off ultrafiltration device (Millipore) and applied on a Superdex 200 (GE Healthcare) size exclusion column equilibrated with PBS. Active fractions were diluted 5 times in phosphate buffer pH 7 and loaded on a MonoQ 5/50 GL column (GE Healthcare). After washing, proteins were eluted with a gradient of 0-0.5 M NaCl. Fractions were analyzed for purity using SDS-PAGE and Coomassie (Instant Blue, Expedeon) staining, and proteins of interest were identified by mass spectrometry.

Antibodies

FITC-conjugated mAbs directed against CD9, CD11a, CD15, CD18, CD31, CD46, CD61, CD62L, CD64, CD66, CD66b, CD99, CD147, HLA-DR, and phycoerythrin (PE)-conjugated mAbs directed against CD35, CD44, CD47, CD49b, CD54, CD58, CD87, CD114, CDw119, CD132, CD151, CD162, and CD321, allophycocyanin (APC)-conjugated mAbs directed against CD11b, CD11c, CD13, CD14, CD29, CD45, CD50, CD55, and Alexa-647-conjugated mAb directed against CD16 were purchased from BD Bioscience. FITC-labeled mAbs against CD120a, CD120b, and CD184, PE-labeled mAbs against CD181, CD182, CD191, and CD192, and APC-conjugated mAbs against JAM-C and Siglec-9 were from R&D Systems. Anti-CD43-FITC and anti-CD141-Alexa-647 were from Santa Cruz Biotechnology. Anti-CD102-FITC, anti-LTB4R-FITC, anti-CD32-PE, and anti-CD89-PE were from AbD Serotec. Anti-CD284-FITC was from Bioconnect. Anti-CD88-PE was from Biolegend. Anti-CD282-PE was from Ebioscience. Anti-CD63-PE was purchased from Immunotech. Fluorescent formylated peptide (fluorescein conjugated of the hexapeptide N-lformyl-Nle-Leu-Phe-Nle-Tyr-Lys) to detect formyl peptide receptor 1 and anti-CD10-APC were purchased from Invitrogen.

Antibody competition assay

Leukocytes from healthy volunteers were isolated as described [23]. Cells were incubated with eluted fractions or purified protein diluted in RPMI (Invitrogen) supplemented with 0.05% human serum albumin (HSA; Sanquin) for 30 min at 37°C. Cells were washed with RPMI-HSA and incubated with different antibodies (Supplementary M&M) as described [23] for 30 min at 4°C. After washing, cells were analyzed using a FACSCalibur (BD biosciences). Neutrophils, monocytes and lymphocytes were selected by gating for forward and side scatter profiles. Where indicated, cells were incubated prior to protease treatment with 0.2 U/ml neuraminidase (*Clostridium perfringens*; Roche) for 45 min at 37°C.

P. aeruginosa transposon mutants

Transposon mutants were obtained from the *Pseudomonas aeruginosa* transposon mutant library [25] and transposon insertion was verified by PCR. Overnight culture supernatant was concentrated using a 30 kD cut-off ultrafiltration device. Concentrated samples were applied on a Superdex 75 (GE Healthcare) column equilibrated with PBS and eluted fractions were analyzed in the PSGL-1 (CD162) antibody competition assay.

Cleavage of cell-surface receptors

Recombinant PSGL-1-Fc, CD44-Fc or CD55 (50 µg/ml; R&D systems) were incubated with IMPa in PBS for 30 min at 37°C. Neuraminidase treatment was performed using 50 µg/ml NanA (from *Streptococcus pneumonia*, kindly provided by G. Taylor) for 30 min at 37°C directly after IMPa treatment. Samples were separated by SDS-PAGE, and proteins were stained with silver.

For Western blot analysis, recombinant PSGL-1-Fc (10 µg/ml) or lymphocytes (CD14 negative leukocytes isolated using the immunomagnetic EasySep CD14 selection kit (Stemcell)), were incubated with IMPa for 30 min at 37°C. Cells were centrifuged and supernatant was collected for analysis. Pelleted cells were lysed in Nonidet P-40 buffer + 10 µM leupeptin and 1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride for 1 h at 4°C. Cell debris was removed by centrifugation and supernatant was collected (cell lysate). Samples were separated by SDS-PAGE, transferred to a PVDF membrane, and blocked with 4% skimmed milk in PBS + 0.05% Tween. Next, blots were incubated with anti-CD43 (1/100) or PSGL-1 antibodies PL1, PL2 and KPL1 (0.5 µg/ml) for 1 h at 37°C. As secondary antibody a HRP-labeled goat anti-mouse IgG (1/10.000, Biorad) was used. Bands were visualized by enhanced chemiluminescence (Amersham).

Hyaluronan/CD44 ELISA

A 96-wells plate (Nunc MaxiSorp) was coated with 100 µg/ml hyaluronan from rooster comb (Sigma) in 0.1 M sodium carbonate buffer pH 9.6 for 1 h at RT. After washing with PBS-Tween, the plate was blocked with 4% skimmed milk in PBS-Tween for 1 h at RT and washed again. CD44-Fc and IMPa were mixed and added to the hyaluronan coated plate for 1 h at 37°C. The plate was washed with PBS-Tween and incubated with HRP-labeled goat anti-human IgG (1/10.000, SouthernBiotech) for 1 h at RT. After washing, the ELISA was developed with TMB as substrate, stopped with H_2SO_4 and optical density at 450 nm was measured.

Static adhesion assay

Static adhesion of neutrophils was performed as described [23] with minor modifications. Calcein-labeled cells ($3x10^5$/ml) were incubated with IMPa for 15 min at 37°C in Hanks balanced salt solution with 0.05% HSA, and subsequently added to the recombinant P-selectin (R&D systems) coated plate for 15 min at RT. Adhered cells were quantified after washing the plates several times with PBS using a platereader fluorometer (FlexStation; Molecular Devices) at 450 nm excitation and 530 nm emission.

Neutrophil rolling

Rolling experiments with neutrophils were performed as described [23]. Neutrophils were pretreated with 10 µg/ml IMPa for 30 min at 37°C, and subsequently perfused over glass

coverslips coated with 10 µg/ml P-selectin/Fc (R&D systems) at a shear stress of 1.6 dyn/cm^2 at 37°C. HUVECs were stimulated with 100 µM histamine (Sigma) for 3 min to induce expression of P-selectin, and neutrophils were immediately perfused at 0.8 dyn/cm^2. Number of adherent cells was visualized using a camera, and recorded images were analyzed using the program Optimas 6.1 (Media Cybergenetics Systems).

Results

Isolation of a PSGL-1 inhibitor from P. aeruginosa supernatant

P. aeruginosa supernatant was monitored for the presence of potential inhibitors of the leukocyte cell-surface receptor PSGL-1 (CD162). Treatment of lymphocytes with concentrated supernatant of *P. aeruginosa* decreased the binding of a CD162 antibody. Activity was maintained after additional purification by ion-exchange and size exclusion chromatography (Fig. 1A). Fractions that inhibited CD162 antibody binding corresponded with a 100 kD band on SDS-PAGE (Fig. 1B). The protein of interest was identified by mass-spec finger printing as PA0572, a hypothetical protein of *P. aeruginosa*. Two other bands of 60 and 40 kD that almost matched with the PSGL-1 inhibitory activity were identified as an intracellular protein involved in energy metabolism and the flagellar hook-associated protein FlgK, respectively.

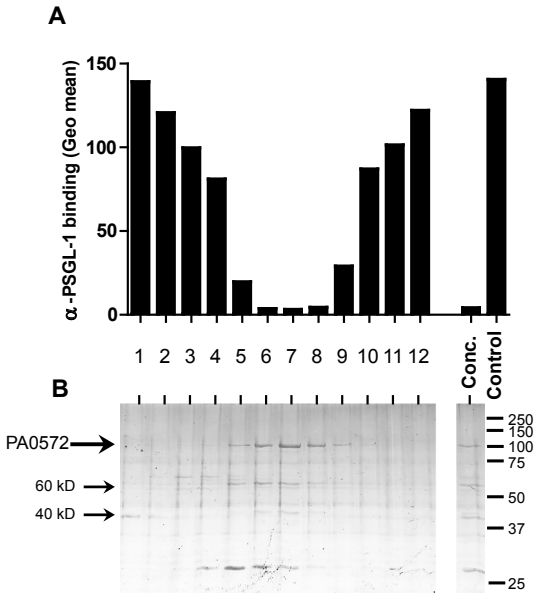

Figure 1. Isolation of a PSGL-1 inhibitor from *P. aeruginosa* supernatant
Supernatant of overnight cultured *P. aeruginosa* (PAO1) was precipitated with ammonium sulfate and separated by ion-exchange and size-exclusion chromatography. (A) Leukocytes were treated with concentrated sample before (conc.) and after separation and fractionation (1-12) by size-exclusion chromatography for 30 min at 37°C. After washing, cells were stained with PE-labeled anti-PSGL-1 (KPL1) for 30 min at 4°C. The geometric mean fluorescence of lymphocytes was measured, by gating for forward- and side-scatter properties. Data are depicted as relative values compared to PSGL-1 staining of untreated cells. (B) The same fractionated samples were loaded on a SDS-PAGE gel and stained with Coomassie. The 100 kD band corresponded with inhibitory activity and was together

Immunomodulating Metalloprotease of Pseudomonas aeruginosa (IMPa)

PA0572 was previously identified in the secretome of *P. aeruginosa* [8], under regulation of LasR. This regulator is involved in expression of several virulence factors of *P. aeruginosa*. Sequence analysis revealed that PA0572 contains a zinc protease motif (GESHELGHNL), which is a conserved proteolytic domain found in zinc metalloproteases. A BLAST search revealed that this hypothetical protease is conserved (over 90%) in all eight sequenced *P. aeruginosa* strains and absent in other *Pseudomonas* species.

A large scale purification with an additional ion exchange step was performed to obtain sufficient pure protein for functional experiments (Fig. 2A). PSGL-1-Fc was incubated with PA0572 to verify its proteolytic activity, resulting in several degradation products (Fig. 2B). Proteolytic activity was blocked in the presence of EDTA, which inhibits metalloproteases. Purified PA0572 dose-dependently decreased binding of anti-CD162 to leukocytes (Fig. 2C-E). Inhibition was more pronounced on lymphocytes (Fig. 2C) in comparison with monocytes and neutrophils (Fig. 2D and 2E). In addition, activity was blocked when incubated at 4°C or in the presence of EDTA. This demonstrates that PA0572, as predicted by sequence analysis, acts as a zinc metalloprotease and shows that proteolytic degradation is responsible for decreased antibody detection of PSGL-1. Inhibition was time-dependent and observed within three minutes after addition of PA0572 (Fig. 2F). Because PSGL-1 plays an essential role in leukocyte homing and extravasation, we named PA0572, Immunomodulating Metalloprotease of *Pseudomonas aeruginosa* (IMPa).

Bacterial supernatant lacking IMPa

To verify that secreted IMPa from *P. aeruginosa* is responsible for PSGL-1 inhibition, we performed experiments with IMPa transposon mutants obtained from the *P. aeruginosa* transposon library [25]. Integration of the transposon in the *impa* gene was confirmed by PCR (data not shown). Concentrated bacterial supernatant, partially purified by size exclusion chromatography of two IMPa mutants strains failed to inhibit PSGL-1 recognition, whereas wild-type showed activity (Fig. 3A). Furthermore, concentrated supernatant of IMPa mutant strain showed a comparable pattern on SDS-PAGE as wild-type except the absence of a 100 kD band (Fig. 3B), which is the size of IMPa in the corresponding active fractions. These data demonstrate the IMPa is responsible for the cleavage of PSGL-1 and corresponds with the presence of the *impa* gene.

Protease specificity

Elastase and alkaline protease of *P. aeruginosa* have both a broad specificity and cleave various host proteins that are involved in antimicrobial defense [4]. IMPa was identified by screening for PSGL-1 inhibitors, however its activity on other cell-surface receptors was not determined. To address its specificity, we treated leukocytes with 3 µg/ml IMPa and stained with a panel of antibodies that recognize different cell-surface receptors involved in innate immunity. Antibody recognition was clearly impaired for CD43 and CD44 (Fig. 4A) on lymphocytes (4-8 fold) and to a smaller extent on monocytes and neutrophils (only inhibition of CD43). Decay accelerating factor (CD55) recognition was inhibited on all three cell types with about 2-fold. The other tested surface receptors were not affected by IMPa (Fig. 4A and Supplementary Fig. 1).

The three additional inhibited (CD43, CD44, CD55) and two unaffected (CD46 and CD31)

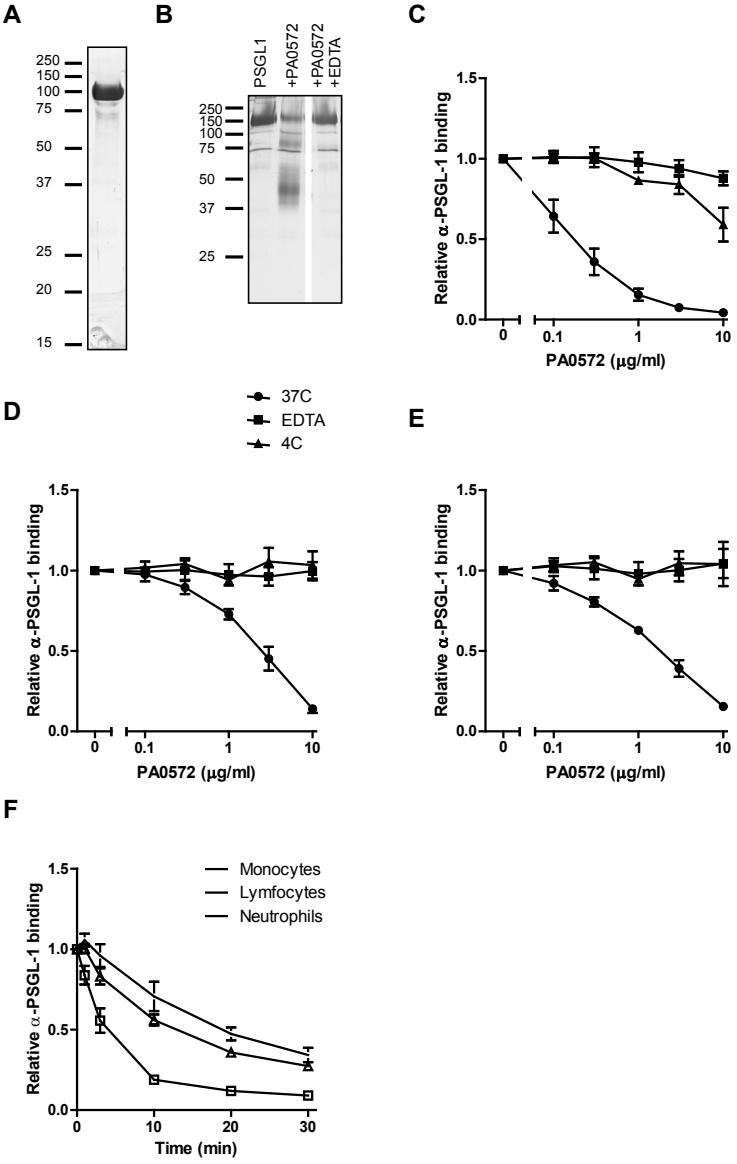

Figure 2. PA0572 dose-dependently cleaves PSGL-1

(A) Purity of PA0572 after large scale purification from *P. aeruginosa* supernatant with an additional MonoQ separation step. (B) Proteolytic activity of PA0572 was confirmed by incubation of PSGL-1-Fc with 3 µg/ml PA0572 +/- 10 mM EDTA. Samples were analyzed by SDS-PAGE and Coomassie staining. (C-E) Leukocytes were treated with various concentration of PA0572 +/- 10 mM EDTA for 30 min at 37°C or at 4°C. After washing, cells were stained with PE-labeled anti-PSGL-1. The relative geometric mean of (C) lymphocytes, (D) monocytes and (E) neutrophils was analyzed by gating for forward- and side-scatter properties. (F) Time-dependent inhibition of PA0572. Leukocytes were incubated with 3 µg/ml PA0572 at 37°C and after 1, 3, 10, 20 or 30 min 10 mM EDTA was added to block proteolytic activity of PA0572, followed by anti-PSGL-1 staining. Data represent the relative geometric mean (compared to control) ± SEM of three independent experiments.

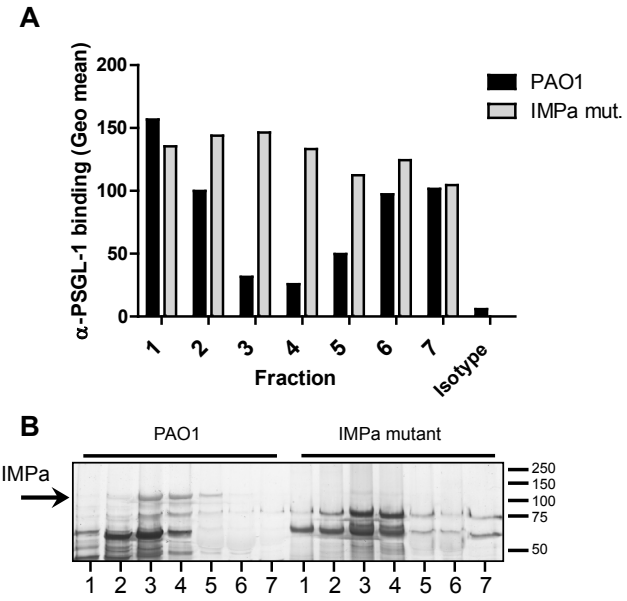

Figure 3. Partially purified supernatant of a *P. aeruginosa* IMPa mutant lacks activity for PSGL-1
Supernatant of *P. aeruginosa* wild-type PAO1 and the IMPa transposon mutant was concentrated and loaded on a Superdex 75 column and fractions of 0.5 ml were collected. (A) Leukocytes were pretreated with gelfiltration fractions of the wild-type and IMPa mutant for 30 min at 37°C and were stained with PE-labeled anti-PSGL-1. Untreated cells were stained with isotype control and anti-PSGL-1. Lymphocytes were gated according to forward- and side-scatter properties and data are expressed as relative geometric mean values compared to PSGL-1 staining of untreated cells. (B) Gelfiltration fractions of PAO1 with inhibitory activity and the corresponding fractions of the IMPa mutant were separated by SDS-PAGE and stained with Coomassie. Arrow indicates the size of IMPa.

in the cell-surface receptor screening were further investigated at various protease concentrations. The dose-response curves of PSGL-1 (CD162) and CD43 on lymphocytes were similar (Fig. 4B), while CD43 inhibition on monocytes (fig. 4C) and neutrophils (Fig. 4D) was less pronounced compared to PSGL-1. CD44 and CD55 inhibition was observed on IMPa-treated lymphocytes, whereas on neutrophils and monocytes only at high concentrations some inhibition could be detected. At the highest dose, IMPa showed no inhibition of CD46 and CD31 staining. Although IMPa inhibition was not restricted to PSGL-1, the protease showed activity for only a small number of tested cell-surface receptors.

IMPa cleavage of CD43 and PSGL-1

To further analyze the cleavage site of IMPa, we incubated recombinant human PSGL-1-Fc with IMPa and performed Western blotting with three antibodies that recognize different parts of PSGL-1. KPL1 (used for screenings assay) and PL1 recognize the N-terminal domain of PSGL-1 and PL2 recognizes a more proximal domain of PSGL-1 [14] (Fig. 5A). The N-terminal epitope was removed after IMPa treatment of recombinant PSGL-1-Fc (Fig. 5B), in contrast PL2 still recognized two degradation products of 50 and 100 kD. Flow cytometric analysis of PSGL-1 cleavage on lymphocytes revealed that the PL2 epitope remained available on IMPa-treated cells (Fig. 5C). These results demonstrate that IMPa removes the N-terminal domain of cell bound PSGL-1, which is essential for the interaction with P-selectin.

Next to PSGL-1, IMPa efficiently inhibited CD43 antibody binding (IC50 of 0.2 µg/ml) especially to lymphocytes (Fig. 4B). Proteolytic degradation of cell-bound CD43 and PSGL-1 was studied in the supernatant and cell lysates of IMPa-treated lymphocytes. Increasing concentrations of IMPa diminished CD43 and PSGL-1 detection in cell lysates, however degradation products of both receptors were not detected in the supernatant (Fig. 5D).

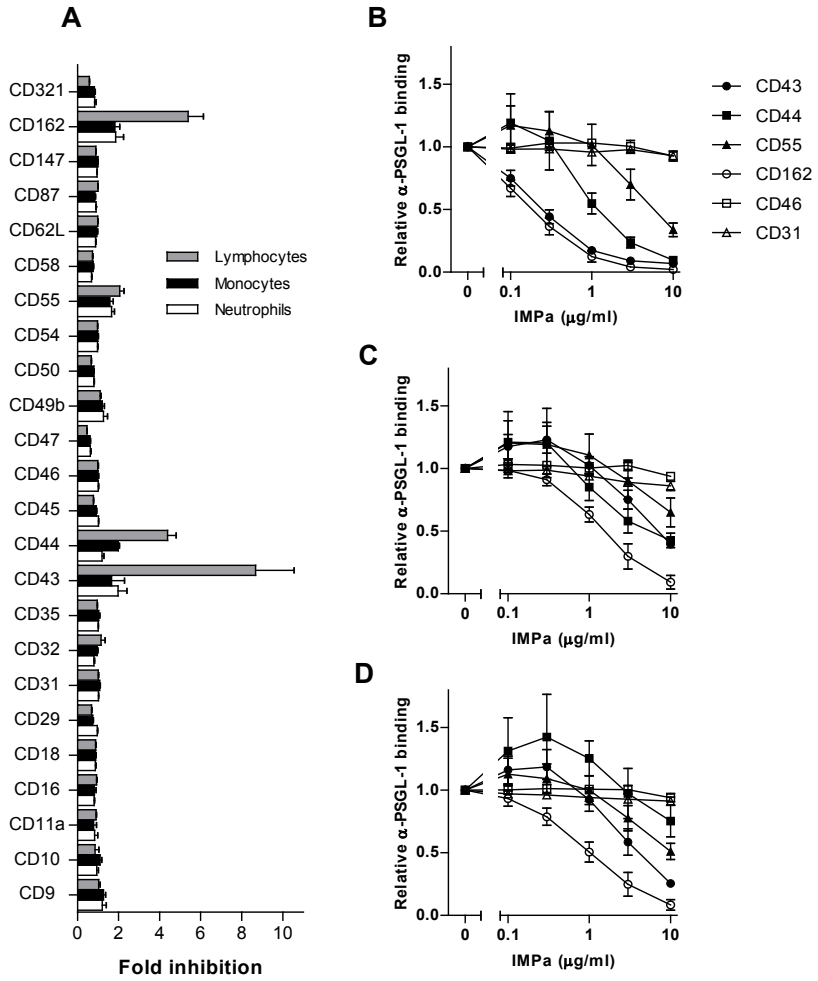

Figure 4. IMPa specificity
(A) Leukocytes were pretreated with 3 µg/ml IMPa for 30 min at 37°C and stained with a panel of fluorescently labeled monoclonal antibodies that recognize different cell-surface receptors of leukocytes. Fold inhibition was calculated by dividing the fluorescence (geometric mean) of untreated cells by that of treated cells. Data represent the mean value ± SEM of three independent experiments. (B-D) Leukocytes were incubated with different concentrations of IMPa for 30 min at 37°C, followed by incubation with the antibodies: CD162-PE, CD44-PE, CD43-FITC, CD55-APC, CD46-PE and CD31-FITC. Lymphocytes (B), monocytes (C) and neutrophils (D) were gated according to forward- and side-scatter properties. Relative fluorescent values were calculated by comparison to untreated cells and represent mean ± SEM of three independent experiments.

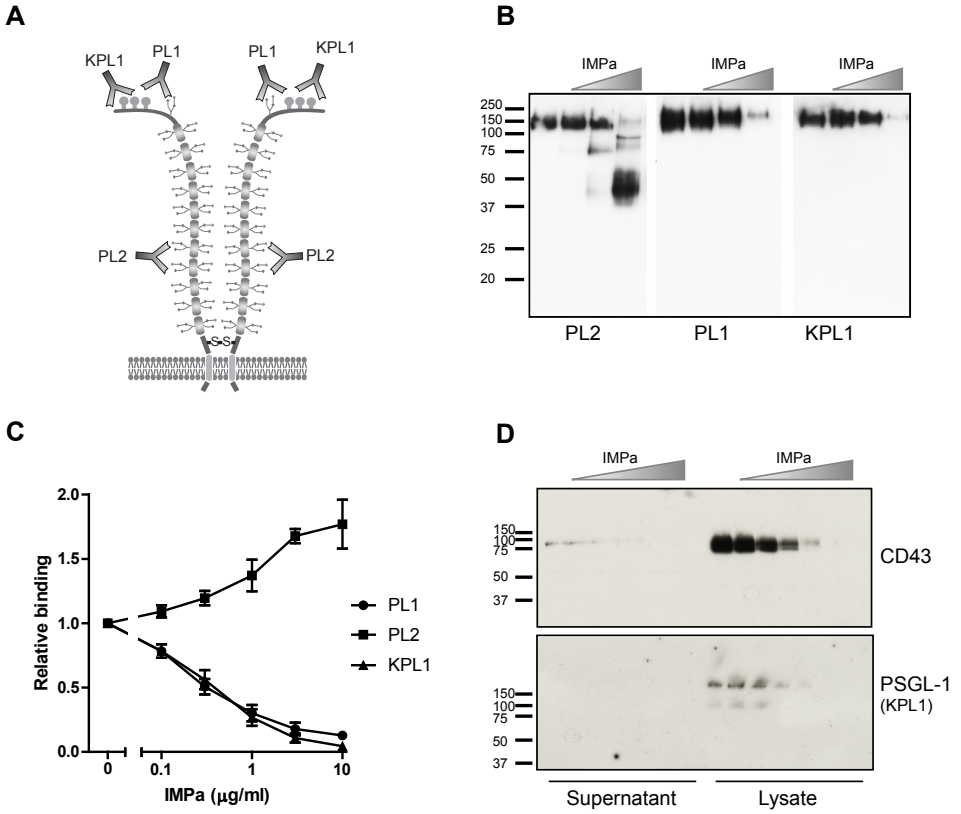

Figure 5. CD43 and PSGL-1 cleavage by IMPa
(A) Schematic representation of dimeric PSGL-1 as expressed on cells. The two antibodies PL1 and KPL1 recognize an epitope in the N-terminal domain of the molecule, whereas PL2 detects an epitope in the central part of PSGL-1. (B) Western blot analysis of PSGL1 cleavage. PSGL-1-Fc was treated with 0, 0.1, 1, and 10 µg/ml IMPa for 30 min at 37°C. Proteins were separated by SDS-PAGE and analyzed by Western blotting. After blocking, blots were stained with PL2, PL1 or KPL1, followed by a HRP-labeled goat anti-mouse IgG. (C) Leukocytes were pretreated with increasing concentrations of IMPa for 30 min at 37°C and stained with the PSGL-1 antibodies PL1, PL2 and KPL1, followed by incubation with a PE-labeled goat anti-mouse IgG. The relative fluorescence was determined by comparison to untreated cells. Data represent the mean ± SEM of three independent experiments. (D) Lymphocytes (CD14 negative leukocytes) were washed with PBS and incubated with 0, 0.1, 0.3, 1, 3, and 10 µg/ml IMPa for 30 min at 37°C. Cell supernatant was collected by centrifugation and cell pellet was lysed by the addition of NP-40 lysis buffer for 1 h at 4°C to obtain cell lysate. Samples were separated by SDS-PAGE and transferred to PVDF membrane. Detection was performed with ECL after staining with anti-CD162 (KPL1) and anti-CD43 followed by a HRP-labeled goat anti-mouse IgG.

IMPa activity is not dependent on sialic acids

Binding of PSGL-1 to P-selectin is dependent on terminal glycan components that include sialic acid and fucose, typified by the sialyl lewis X (sLex) determinant. Naïve lymphocytes cannot bind to P-selectin, as they lack fucosyltransferase 7 (FuT7), necessary for proper sLex synthesis [15]. Both removal of sialic acids by neuraminidase and disruption of FuT7 eliminates P-selectin binding. In view of the fact that IMPa cleaves cell-surface receptors more efficient on lymphocytes in comparison with monocytes and neutrophils, we investigated the activity of IMPa after removal of sialic acid by neuraminidase. Treatment

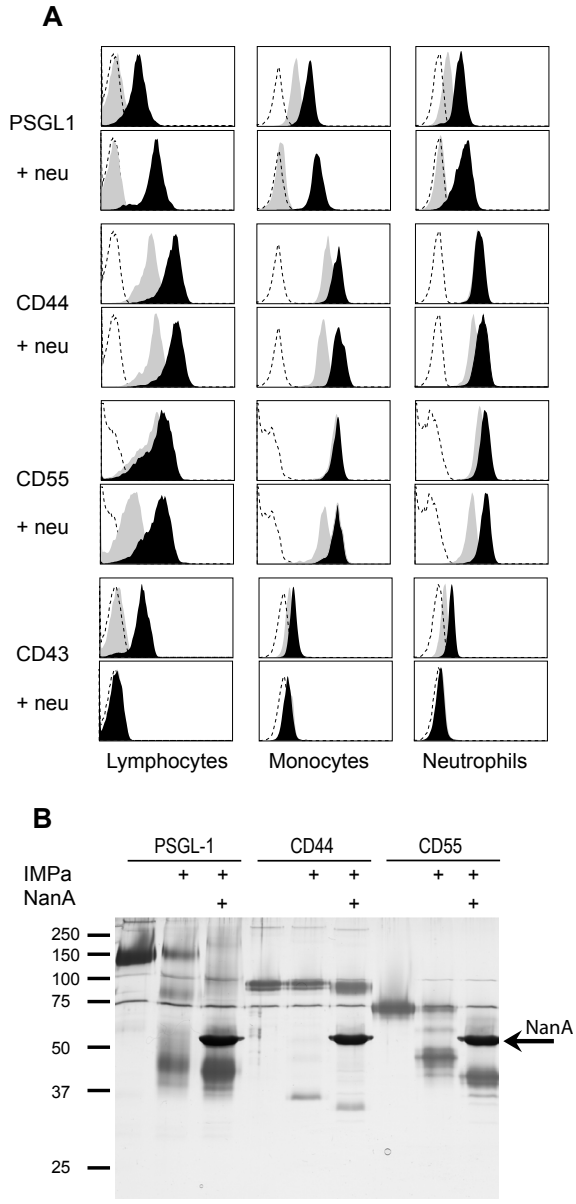

Figure 6. Effect of neuraminidase on IMPa specificity
(A) Leukocytes were incubated with 0.2 U/ml neuraminidase from *Clostridium perfringens* for 45 min at 37°C. Followed by treatment with 1 µg/ml IMPa for 30 min at 37°C. Cells were stained with CD43-FITC, CD44-PE, CD162-PE and CD55-APC for 30 min at 4°C. Histograms show binding of the different antibodies to lymphocytes, monocytes and neutrophils that were gated according to forward- and side-scatter characteristics. Histograms represent untreated cells (black filled histograms), IMPa-treated cells (grey filled histograms), and unstained cells (dotted line). (B) Recombinant PSGL-1-Fc, CD44-Fc and CD55 all 50 µg/ml were incubated with 3 µg/ml IMPa. Next, for neuraminidase treatment, 50 µg/ml NanA was added for 30 min at 37°C. Cleavage was analyzed by SDS-PAGE and silver staining.

of cells with neuraminidase was effective as illustrated by impaired binding of anti-CD43, which recognizes a neuraminidase-sensitive epitope (Fig. 6A). Pretreatment of neutrophils and monocytes with neuraminidase enhanced the IMPa activity towards PSGL1, as binding of anti-PSGL1 was completely lost (Fig. 6A). Removal of sialic acids also clearly enhanced the activity for CD55 on all cell types, whereas cleavage of CD44 was unaffected. Recombinant CD44-Fc, CD55 and PSGL-1-Fc were incubated with IMPa in the presence of neuraminidase to investigate the effect of sialic acids on proteolytic activity. IMPa degraded PSGL-1 and CD55 efficiently, whereas a relative small part of CD44 was cleaved in the absence of neuraminidase (Fig. 6B). Removal of sialic acids enhanced CD55 and PSGL-1 cleavage, and the molecular weight of CD44 and CD55 degradation products slightly decreased. In conclusion, removal of sialic acids by neuraminidase seems to enhance the IMPa activity.

Functional inhibition by IMPa

CD44 expressed on leukocytes interacts with the extracellular matrix component hyaluronan. Binding of recombinant CD44-Fc to a hyaluronan coated surface was inhibited in the presence of IMPa (Fig. 7A). To investigate whether IMPa also interferes with PSGL-1/P-selectin interaction, we measured the adhesion of IMPa-treated neutrophils to a P-selectin coated surface under static conditions. Adhesion of neutrophils was blocked by the anti-PSGL-1 monoclonal antibody KPL1. IMPa reduced the adhesion of neutrophils with 50% at 0.1 µg/ml and completely abolished adhesion at 3 µg/ml protease (Fig. 7B). The impaired interaction of PSGL-1 and CD44 with their ligands suggests that degradation of these receptors by IMPa has functional consequences.

In the bloodstream, PSGL-1 initiates rolling of leukocytes by transient interactions with P-selectin on endothelial cells. These conditions were mimicked using a flow chamber in which neutrophils were perfused over glass cover slips coated with P-selectin-Fc or histamine-activated human umbilical vein endothelial cells (HUVECs). After 5 min, the number of rolling neutrophils was determined by measuring the number of adhered cells per square millimeter. Pre-incubation of neutrophils with anti-PSGL-1 (KPL1) impaired rolling adhesion with at least 80% over a P-selectin-Fc coated surface and HUVECs, demonstrating that rolling was dependent on PSGL-1 (Fig. 7C and 7D). In the presence of 10 µg/ml IMPa rolling adhesion was diminished with 73% on the P-selectin-Fc coated surface and 63% over activated HUVECs (Fig. 7C and 7D). In conclusion, IMPa potently inhibits binding of PSGL-1 under static and flow conditions.

Discussion

Pseudomonas aeruginosa secretes several proteases that degrade proteins of the host immune system and extracellular matrix components. In the present study, we isolated a yet uncharacterized metalloprotease, IMPa (PA0572), that shows proteolytic activity towards four glycosylated leukocyte surface receptors. Homology search revealed that IMPa is only present in *P. aeruginosa* and absent in all other *Pseudomonas* species. IMPa homologs are found in *Vibrio* and *Shewanella* that also belong to the gammaproteobacteria. However the conservation at protein level is rather low, around or below 30%. All these homologs are hypothetical proteins containing a signal sequence and a zinc metalloprotease motif.

The IMPa-sensitive cell-surface receptors CD43, CD44 and PSGL-1 are abundantly O-linked

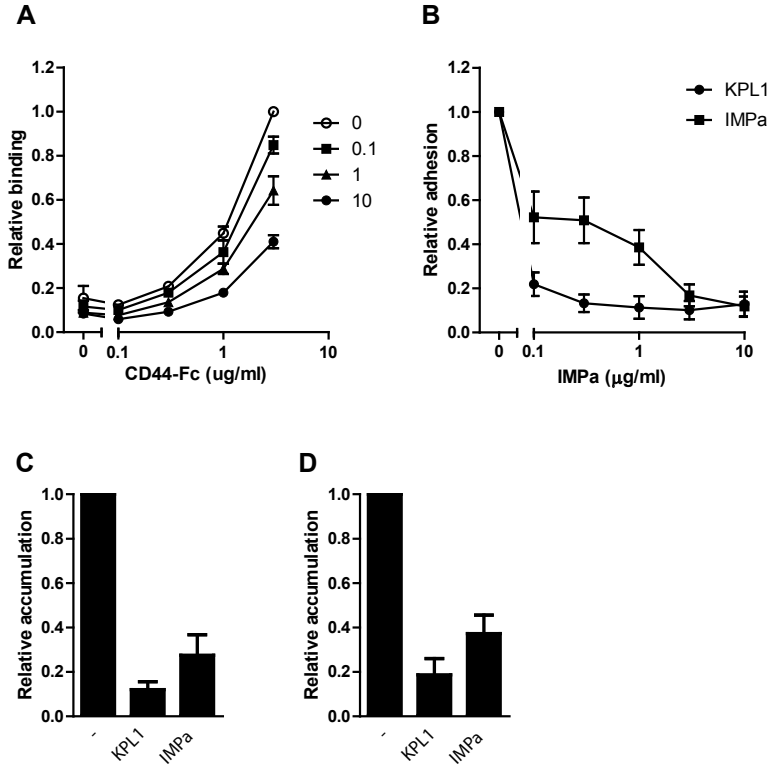

Figure 7. Functional effects of IMPa
(A) An ELISA plate was coated with 100 µg/ml hyaluronan for 1 h at RT. Plate was blocked with 4% skimmed milk in PBS-Tween for 1 h at RT. After washing, CD44-Fc was incubated with IMPa in 1% skimmed milk in PBS-Tween and directly added to the 96-wells plate for 1 h at 37°C. After washing, HRP-labeled goat anti-human (1/10.000) in 1% skimmed milk in PBS-Tween was added to the wells for 1 h at RT. TMB was used as substrate and the absorbance at 450 nm was determined. Data represent mean ± SEM of three independent experiments. (B) An ELISA plate was coated with 3 µg/ml P-selectin and blocked with 4% skimmed milk in PBS. Neutrophils were labeled with 4 µM calcein-AM for 20 min at RT. Cells ($3x10^5$/well) were pretreated with IMPa for 15 min at 37°C before addition to the plate for 15 min at RT. Adherence to P-selectin was determined by measuring the fluorescence after several washes with PBS. Data represent the mean values ± SEM of three independent experiments. (C) Neutrophils were treated with 10 µg/ml IMPa or KPL1 (anti-PSGL-1) as a positive control for 30 min at 37°C, and subsequently perfused over P-selectin-Fc coated glass cover slips with a shear stress of 1.6 dyn/cm^2 for 5 min at 37°C. After washing for 1 min, accumulated neutrophils were quantified. (D) Isolated HUVECs were cultured on glass cover slips and stimulated with 100 µM histamine for 3 min. Neutrophils were preincubated with 10 µg/ml IMPa or KPL1 for 30 min at 37°C, and subsequently perfused at 0.8 dyn/cm^2 for 5 min at 37°C over activated HUVECs. After washing for 1 min, accumulated neutrophils were quantified. Data represent relative accumulation of neutrophils compared to control cells and are mean values ± SEM of at least three independent experiments.

glycosylated, suggesting that IMPa activity is dependent on O-linked glycosylation. The same degree of specificity for highly O-glycosylated proteins was described for the secreted metalloprotease of *Pasteurella haemolytica*. This protease shows high specificity for O-glycosylated cell-surface receptors, including CD43 and CD44, whereas it cannot cleave N-glycosylated proteins or nonglycosylated proteins [26,27]. The glycosylation status of PSGL-1 differs between leukocytes. In contrast to naive lymphocytes, neutrophils and monocytes express the fucosyltransferase 7, necessary for proper sLe^x synthesis and P-selectin binding. Activation of lymphocytes induces FuT7 expression, resulting in proper

glycosylation of PSGL-1 to interact with P-selectin [15]. We show higher activity of IMPa for CD43 and PSGL-1 expressed on lymphocytes in comparison to neutrophils and monocytes. This suggests that additional glycosylation, embodied by the tetrasaccharide sLe^x, impairs IMPa activity. Indeed, the cleavage efficacy of IMPa for CD55 and PSGL-1 on neutrophils and monocytes, was enhanced after removal of sialic acid by neuraminidase. Thus, a functional sLe^x moiety seems to hamper IMPa activity. Comparable higher protease activities towards CD43 after removal of sialic acids by neuraminidase are described for pancreatic elastase and V8 protease of *Staphylococcus aureus* [28]. In contrast to IMPa and the above described proteases, the activity of the secreted metalloprotease of *Pasteurella haemolytica*, which also degrades CD43 and CD44 [26], was lost upon neuraminidase treatment [27]. The same has been described for the activity of SSL5 of *S. aureus*, although SSL5 is not a protease. SSL5 interacts and functionally inhibits PSGL-1 in a sLe^x dependent way, as desialylation of PSGL-1 completely blocked SSL5 binding and activity [23]. Therefore, in contrast to IMPa, SSL5 shows activity towards neutrophils and monocytes, but not towards PSGL-1 expressed on naïve lymphocytes.

P. aeruginosa itself produces neuraminidases, that are involved in respiratory infection and biofilm formation [29]. Mutant strains that lack neuraminidase fail to colonize the respiratory tract, however are fully virulent when introduced intravenously. Desialylation of mucosal surfaces increases the adherence and contributes to the colonization of *P. aeruginosa* [30]. The gene encoding neuraminidase is among the most conserved and highest expressed in *P. aeruginosa* strains isolated from cystic fibrosis patients [31] and increases biofilm formation. Production of neuraminidase by *P. aeruginosa* in the respiratory tract can facilitate removal of sialic acids from leukocyte receptors. This will increase the susceptibility of these receptors to subsequent cleavage by IMPa, especially on neutrophils.

The secreted proteases elastase, alkaline protease, LasA and protease IV of *P. aeruginosa* all target multiple proteins [4,32,33]. The cleavage efficiency widely varies between different substrates of pseudomonal proteases. At high concentrations, these proteases cleave many different proteins, however the biological relevance is questionable. IMPa production is regulated via the regulator LasR of the *las* quorum sensing system [8]. The expression of LasR is dependent on culture conditions [34]. For instance, the expression of LasR and alkaline protease was enhanced in *P. aeruginosa* grown in the presence of sputum from cystic fibrosis patients. The production of IMPa by *P. aeruginosa* in overnight culture supernatant was sufficient to successfully purify the protease and in this concentration range IMPa displays proteolytic activity and immune evasive features. Three leukocyte receptors (CD43, CD44 and PSGL-1) that were identified in the IMPa target screenings assay interact with selectins. Removing these receptors form the surface of leukocytes impairs the collective rolling adhesion and consequently transmigration to the site of infection as demonstrated in our rolling experiments with neutrophils. In this way, *P. aeruginosa* can prevent recruitment and activation of cells of the immune system and thereby increases its survival in the host. These findings suggest a role for IMPa in *P. aeruginosa* pathogenesis and persistence that can be addressed by studying IMPa knockouts in vivo.

Footnotes

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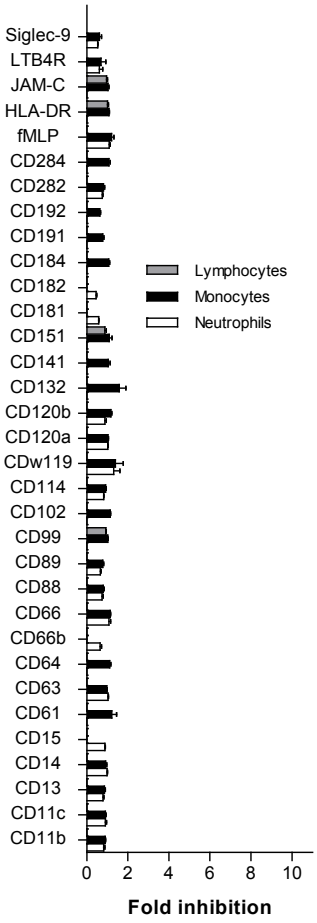
References

1. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318-1322.
2. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, et al. (2001) Gene expression in Pseudomonas aeruginosa biofilms. Nature 413: 860-864.
3. Young LS, Armstrong D (1972) Human immunity to Pseudomonas aeruginosa. I. In-vitro interaction of bacteria, polymorphonuclear leukocytes, and serum factors. J Infect Dis 126: 257-276.
4. Matsumoto K (2004) Role of bacterial proteases in pseudomonal and serratial keratitis. Biol Chem 385: 1007-1016.
5. Hong YQ, Ghebrehiwet B (1992) Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol 62: 133-138.
6. Wolz C, Hellstern E, Haug M, Galloway DR, Vasil ML, et al. (1991) Pseudomonas aeruginosa LasB mutant constructed by insertional mutagenesis reveals elastolytic activity due to alkaline proteinase and the LasA fragment. Mol Microbiol 5: 2125-2131.
7. Gambello MJ, Kaye S, Iglewski BH (1993) LasR of Pseudomonas aeruginosa is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. Infect Immun 61: 1180-1184.
8. Nouwens AS, Beatson SA, Whitchurch CB, Walsh BJ, Schweizer HP, et al. (2003) Proteome analysis of extracellular proteins regulated by the las and rhl quorum sensing systems in Pseudomonas aeruginosa PAO1. Microbiology 149: 1311-1322.
9. Tang HB, DiMango E, Bryan R, Gambello M, Iglewski BH, et al. (1996) Contribution of specific Pseudomonas aeruginosa virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. Infect Immun 64: 37-43.
10. Vestweber D, Blanks JE (1999) Mechanisms that regulate the function of the selectins and their ligands. Physiol Rev 79: 181-213.
11. Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, et al. (1992) Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J Cell Biol 118: 445-456.
12. Somers WS, Tang J, Shaw GD, Camphausen RT (2000) Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1. Cell 103: 467-479.
13. Xia L, Sperandio M, Yago T, McDaniel JM, Cummings RD, et al. (2002) P-selectin glycoprotein ligand-1-deficient mice have impaired leukocyte tethering to E-selectin under flow. J Clin Invest 109: 939-950.
14. Epperson TK, Patel KD, McEver RP, Cummings RD (2000) Noncovalent association of P-selectin glycoprotein ligand-1 and minimal determinants for binding to P-selectin. J Biol Chem 275: 7839-7853.
15. Carlow DA, Gossens K, Naus S, Veerman KM, Seo W, et al. (2009) PSGL-1 function in immunity and steady state homeostasis. Immunol Rev 230: 75-96.
16. Katayama Y, Hidalgo A, Chang J, Peired A, Frenette PS (2005) CD44 is a physiological E-selectin ligand on neutrophils. J Exp Med 201: 1183-1189.
17. Jiang D, Liang J, Noble PW (2007) Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 23: 435-461.
18. Fukuda M, Tsuboi S (1999) Mucin-type O-glycans and leukosialin. Biochim Biophys Acta 1455: 205-217.
19. Matsumoto M, Atarashi K, Umemoto E, Furukawa Y, Shigeta A, et al. (2005) CD43 functions as a ligand for E-Selectin on activated T cells. J Immunol 175: 8042-8050.
20. Carlow DA, Ziltener HJ (2006) CD43 deficiency has no impact in competitive in vivo assays of neutrophil or activated T cell recruitment efficiency. J Immunol 177: 6450-6459.
21. Yago T, Fu J, McDaniel JM, Miner JJ, McEver RP, et al. (2010) Core 1-derived O-glycans are essential E-selectin ligands on neutrophils. Proc Natl Acad Sci U S A 107: 9204-9209.
22. Rooijakkers SH, van Kessel KP, van Strijp JA (2005) Staphylococcal innate immune evasion. Trends Microbiol 13:

596-601.
23. Bestebroer J, Poppelier MJ, Ulfman LH, Lenting PJ, Denis CV, et al. (2007) Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 109: 2936-2943.
24. Bestebroer J, van Kessel KP, Azouagh H, Walenkamp AM, Boer IG, et al. (2009) Staphylococcal SSL5 inhibits leukocyte activation by chemokines and anaphylatoxins. Blood 113: 328-337.
25. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, et al. (2003) Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 100: 14339-14344.
26. Sutherland DR, Abdullah KM, Cyopick P, Mellors A (1992) Cleavage of the cell-surface O-sialoglycoproteins CD34, CD43, CD44, and CD45 by a novel glycoprotease from Pasteurella haemolytica. J Immunol 148: 1458-1464.
27. Abdullah KM, Udoh EA, Shewen PE, Mellors A (1992) A neutral glycoprotease of Pasteurella haemolytica A1 specifically cleaves O-sialoglycoproteins. Infect Immun 60: 56-62.
28. Remold-O'Donnell E, Rosen FS (1990) Proteolytic fragmentation of sialophorin (CD43). Localization of the activation-inducing site and examination of the role of sialic acid. J Immunol 145: 3372-3378.
29. Soong G, Muir A, Gomez MI, Waks J, Reddy B, et al. (2006) Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. J Clin Invest 116: 2297-2305.
30. Cacalano G, Kays M, Saiman L, Prince A (1992) Production of the Pseudomonas aeruginosa neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. J Clin Invest 89: 1866-1874.
31. Lanotte P, Watt S, Mereghetti L, Dartiguelongue N, Rastegar-Lari A, et al. (2004) Genetic features of Pseudomonas aeruginosa isolates from cystic fibrosis patients compared with those of isolates from other origins. J Med Microbiol 53: 73-81.
32. Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ (1998) Protease IV, a unique extracellular protease and virulence factor from Pseudomonas aeruginosa. J Biol Chem 273: 16792-16797.
33. Spencer J, Murphy LM, Conners R, Sessions RB, Gamblin SJ (2010) Crystal structure of the LasA virulence factor from Pseudomonas aeruginosa: substrate specificity and mechanism of M23 metallopeptidases. J Mol Biol 396: 908-923.
34. Duan K, Surette MG (2007) Environmental regulation of Pseudomonas aeruginosa PAO1 Las and Rhl quorum-sensing systems. J Bacteriol 189: 4827-4836.



Supplementary figure 1. IMPa specificity
Leukocytes were pretreated with 3 µg/ml IMPa for 30 min at 37°C and stained with a panel of fluorescently labeled monoclonal antibodies that recognize different cell-surface receptors of leukocytes. Fold inhibition was calculated by dividing the fluorescence of treated cells by that of untreated cells. Data represent the mean value ± SEM of three independent experiments.

6

Evasion of Toll-like Receptor 2 Activation by Staphylococcal SSL3

Bart Bardoel[a], Tim Bouman[a], Piet Aerts[a], Eric Huizinga[b], Harma Brondijk[b], Jos van Strijp[a], and Carla de Haas[a]

[a] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

[b] Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht, The Netherlands

Abstract

Toll-like receptors (TLRs) are crucial for our host defense against microbial infections. TLR2 is especially important to fight bacterial infections, since it specifically recognizes bacterial lipoproteins of both Gram-positive and Gram-negative origin. Present on a variety of immune cells, TLR2 mediates bacterial eradication in the initial phase of infection and induces an adaptive immune response important for protection in later infection phases.

Staphylococcus aureus is a leading human pathogen that causes increasing healthcare problems due to its increased resistance to antibiotics. *S. aureus* secretes a wide variety of proteins that inhibit innate immunity. Recently, several immune evasive functions of staphylococcal superantigen-like proteins (SSLs) have been identified. Here we describe that SSL3 binds and potently inhibits TLR2 activation of human neutrophils and monocytes, and also efficiently inhibits IL-8 production of MALP-2/Pam3Cys stimulated HEK-TLR2 cells. Moreover, SSL3 binds and inhibits activation of murine TLR2. SSL4 shares a high homology with SSL3 and slightly inhibited TLR2 activation, whereas other SSLs showed no TLR2 activity. SSL3 is the first described bacterial protein that interferes with TLR2 activation by direct interaction with this receptor.

Introduction

Our host immune response critically depends on pattern-recognition receptors, molecules that discriminate foreign antigens from self.-antigens Toll-like receptors (TLRs) play a crucial role in the detection of microbial infections from mammals to insects. TLRs have evolved to recognize highly conserved structures of viral (TLR 3, 7, 8, and 9) [1] and bacterial (TLR1, 2, 4, 5, 6, 7, and 9) origin [2]. This specificity allows TLRs to rapidly detect the presence of an invading microorganism and subsequently induce activation of inflammatory and antimicrobial innate immune responses [3,4,5]. In addition, TLRs expressed on dendritic and B cells initiate antigen-specific adaptive immune responses [6,7,8,9].

TLR ligands range from bacterial lipoproteins (TLR2), lipopolysaccharide (TLR4) and flagellin (TLR5) to bacterial CpG-rich DNA (TLR9) and double (TLR3) or single stranded RNA (TLR7 and 8) [3,10]. TLRs are type I transmembrane glycoproteins characterized by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor (TIR) domain. All TLRs (except TLR3) use MyD88 as an universal adapter protein to activate the transcription factor NF-κB. Ligand-induced dimerization of TLRs is believed to trigger recruitment of MyD88 to the intracellular TIR domains to initiate signalling [11]. Most TLRs form homodimers upon ligand binding. In contrast, TLR2 forms heterodimers with either TLR1 (TLR1/2) or TLR6 (TLR2/6) to adequately respond to tri- and diacylated lipoproteins, respectively [12]. These di- and tri-acylated modifications of a N-terminal cysteine residue are conserved and important to anchor hydrophilic proteins to the bacterial membrane [13]. These cysteine modifications are essential to trigger TLR2 activation as demonstrated by the diacylated lipopeptide Pam2Cys, which is a derivative of macrophage-activating lipopeptide (MALP-2) of *Mycoplasma fermentans* [14]. Gram-positive bacteria contain diacylated lipoproteins, whereas lipoproteins of Gram-negative bacteria have an additional acyl group [15]. A wide variety of other ligands have been described for TLR2, however these are usually less potent and it was suggested that activity of these putative TLR2 ligands was due to contamination with highly active lipoproteins or lipopeptides [16].

TLR2 has been shown to be critical for host protection against several bacterial infections including *Staphylococcus aureus* (*S. aureus*) [17,18,19,20,21]. Strains deficient in diacyl modification of lipoproteins fail to activate TLR2 *in vivo*, resulting in a reduced inflammatory response [22]. *S. aureus* causes a wide variety of infections ranging from mild skin infections to life-threatening diseases such as pneumonia, meningitis, endocarditis, and sepsis. There is an increasing resistance of *S. aureus* to antibiotics. Therefore, more insight is needed into the pathophysiology of *S. aureus* infections for development of additional therapeutic strategies. The complex and versatile lifestyle of *S. aureus* is due to its diverse array of virulence factors that enable adherence and invasion of different sites of the body.

In the last decade, it has become clear that *S. aureus* also produces a large array of small secreted proteins that interact with various components of the innate immune system [23]. Recently, we have been investigating the staphylococcal superantigen-like proteins 1 to 11 (SSL1 to 11) located on pathogenicity island SAPI2. Although SSL proteins have sequence homology to bacterial superantigens, they lack superantigenic properties and their function was long unknown [24]. We and other have shown that SSL proteins are potent immune evasion molecules that specifically interact with various parts of the innate immune system. SSL5 binds P-selectin glycoprotein ligand-1 (PSGL1) and inhibits rolling of neutrophils across the endothelial lining [25,26]. In addition, SSL5 inhibits phagocyte activation by binding all

chemokine and anaphylatoxin receptors [27,28]. SSL7 binds IgA and complement component C5, and thereby prevents activation of C5 into the chemotactic C5a [29]. SSL10 binds and inhibits chemokine receptor CXCR4 [30] and human IgG [31,32]. Thus far, the functions of the other SSLs are unknown, but we hypothesize that they also have a role in immune evasion. In this study, we expressed SSL3 and screened whether it interacts with cell-surface proteins of leukocytes. We identified that SSL3 binds and potently inhibits TLR2 activation.

Materials and methods

Antibodies

FITC-conjugated mAbs directed against CD9, CD11a, CD31, CD46, CD62L, CD66, and phycoerythrin (PE)-conjugated mAbs directed against CD35, CD44, CD47, CD49b, CD54, CD58, CD87, CD114, CDw119, CD162, and CD321, allophycocyanin (APC)-conjugated mAbs directed against CD11b, CD11c, CD13, CD14, CD29, CD45, CD50, CD55, and Alexa-647-conjugated mAb directed against CD16 were purchased from BD Bioscience. FITC-labeled mAbs against CD120a, and CD120b, and an APC-conjugated mAb against Siglec-9 were from R&D Systems. Anti-CD43-FITC was from Santa Cruz Biotechnology. Anti-LTB4R-FITC, anti-CD32-PE, and anti-CD89-PE were from AbD Serotec. Anti-CD88-PE was from Biolegend. Anti-CD282-PE was from Ebioscience. Anti-CD63-PE was purchased from Immunotech. Fluorescent formylated peptide (fluorescein conjugated of the hexapeptide N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys) to detect formyl peptide receptor 1 and anti-CD10-APC were purchased from Invitrogen.

Cloning, expression and purification of SSL3 and 4

For expression of recombinant SSL3, the SSL3 gene of *S. aureus* strain NCTC 8325 (SAOUHSC_00386), except for the signal sequence, was cloned into the pRSETB vector (Invitrogen) as described [25]. After verification of the correct sequence, the pRSETB/SSL3 expression vector was transformed in Rosetta-Gami(DE3)pLysS *E. coli* (Novagen). Expression of histidine (His)-tagged SSL3 was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Roche Diagnostics) for 4 h at 37°C in LB containing 20 mM glucose. His-tagged SSL3 was isolated under denaturating conditions on a HiTrap chelating column, according to the manufacturer's description. Elution was performed in 50 mM EDTA under denaturating conditions. Renaturation of His-SSL3 was performed by dialysis, after which the His-tag was removed by enterokinase cleavage according to the manufacturer's instructions (Invitrogen). Finally, the purity of SSL3 was checked by SDS-PAGE and protein was stored in PBS at -20°C. Cloning and expression of SSL 1, 2, 4, and 5 to 11 of *S. aureus* strains NCTC 8325 and SSL4 of MRSA252 was performed as described for SSL3 with minor modifications. The N-terminal histidine tag of the pRSETB vector, contains besides the histidine tag and enterokinase cleavage site also an Xpress epitope, which was replaced by a 6 residue histidine tag just downstream the enterokinase cleavage site. After enterokinase cleavage, an additional glycine residue remains at the N-terminus of the SSL4 proteins.

Cells

Human neutrophils and peripheral mononuclear cells (PBMCs) were isolated as described [25]. Human embryonal kidney cells expressing TLR2 (HEK-TLR2) and TLR2 in combination

with TLR1 (HEK-TLR1/2) and TLR6 (HEK-TLR2/6) were obtained from Invivogen. HEK-TLR cell lines were maintained in DMEM, containing 10 µg/ml gentamicin, 10 µg/ml blasticidin and 10% FCS. Mouse macrophage cell line RAW264.7 was cultured in DMEM, containing 10 µg/ml gentamicin and 10% FCS.

SSL3 binding to cells

To determine binding of SSL3 to different leukocyte populations, SSL3 was labeled with fluorescein isothiocyanate (FITC). Therefore, 1 mg/ml SSL3 was incubated with 100 µg/ml FITC in 0.1 M sodium carbonate buffer (pH 9.6) for 1 hour at 37°C. A HiTrap desalting column (GE healthcare) was used to separate FITC-labeled SSL3 from unbound FITC. For binding of SSL3-FITC to leukocytes, human neutrophils (5×10^6 cells/ml) and PBMCs (1×10^7 cells/ml) were incubated on ice for 30 min with increasing concentrations of SSL3-FITC in RPMI (Gibco), containing 0.05% human serum albumin (Sanquin). After washing, fluorescence was measured on a flow cytometer (FACSCalibur; Becton Dickinson).

Competition for receptor binding

To determine a putative receptor for SSL3, a mixture of neutrophils (5×10^6 cells/ml) and PBMCs (1×10^7 cells/ml) were incubated with either SSL3 (10 µg/ml) or RPMI/HSA and incubated 30 min on ice. Subsequently, 39 different FITC-, PE-, or APC-conjugated monoclonal antibodies (mAbs) directed against various cell-surface receptors were added to the cell mixture and incubated for 45 min on ice. After washing, fluorescence was measured using flow cytometry. Neutrophils, monocytes and lymphocytes were selected by gating. In another experiment, leukocytes were incubated with increasing concentrations of SSL3 for 30 min at 4°C. Subsequently, the cells were incubated with anti-TLR2 antibody (anti-CD282-PE; 1:100 dilution) using the same conditions as in the screenings assay.

TLR2 ligand-induced IL-8 production

To test the effect of SSL3 on TLR2 ligand-induced IL-8 production, HEK-TLR2, HEK-TLR1/2, HEK-TLR2/6, PBMC, neutrophils, and RAW264.7 cells were used. HEK and RAW264.7 cells were seeded in 96 wells culture plates until confluency. Freshly isolated PBMC and neutrophils were added to 96 wells culture plates (2.5×10^6 cells/well). To avoid activation of TLR4 on PBMC and neutrophils by endotoxin, SSL3 and recombinant AprA of *P. aeruginosa* (Chapter 2) were pretreated with 20 µg/ml polymyxin B sulfate (Sigma) for 1 hour. Additionally, PBMC were preincubated with 10 µg/ml blocking anti-TLR4 mAb (clone HTA125; Bioconnect) for 30 minutes. Next, the cells were preincubated for 30 minutes at 37°C with increasing concentrations of SSL3. Then, cells were stimulated with increasing concentrations of Pam2Cys, Pam3Cys (both from EMC microcollections), MALP-2 (Santa Cruz), or recombinant flagellin of *P. aeruginosa* (Chapter 2), as indicated in the results section. After overnight incubation in a 37°C incubator, culture supernatants were tested for presence of IL-8 using a specific ELISA following the manufacturer's instructions (Sanquin). Cultures supernatants of RAW264.7 cells were tested for the presence of mouse TNFα using a specific ELISA kit (R&D systems). IL-8 production experiments with PBMC and neutrophils were performed in RPMI/10% FCS. Experiments with HEK and RAW264.7 cells were performed in DMEM/10% FCS. Cytotoxic effect of SSL3 on cells was tested using the lactate dehydrogenase (LDH) cellular cytotoxicity detection kit following the manufacturer's

description (Roche Diagnostics). In some experiments, next to SSL3, SSL4 and the other SSLs of SAPI2 were tested on IL-8 production by MALP-2-activated HEK-TLR2/6 cells, as described above.

Cloning and expression of human and mouse TLR2

The recombinant extracellular domain of human TLR2 (hTLR2) was cloned in HEK293 cells (U-Protein Express, The Netherlands). The recombinant extracellular domain of mouse TLR2 (mTLR2) was cloned and expressed by Dr. Harma Brondijk (Crystal and Structural Chemistry, University Utrecht, The Netherlands) in HEK293 cells. Both hTLR2 and mTLR2 contain a N-terminal 6 residues histidine tag, a 3x streptavidin tag and a TEV cleavage site.

ELISA

To test binding of SSL3 to the recombinant extracellular domains of human and mouse TLR2, the TLR2 proteins were coated to an ELISA plate (Nunc maxisorp) at 10 µg/ml. Wells were blocked with 4% skimmed milk in PBS/0.05% Tween. His-tagged SSL3 was allowed to bind to the coated TLR2 proteins for 1 hour at 37°C. Bound His-SSL3 was detected with anti-Xpress mAb (Invitrogen) and subsequent binding of peroxidase-labeled goat anti-mouse IgG and visualized as described [33].

Results

SSL3 binds to neutrophils and monocytes via TLR2

To investigate its role in immune evasion, we cloned and expressed SSL3 of *S. aureus* strain NCTC 8325 in *E. coli*. The protein was pure according to SDS-PAGE and fluorescently-labelled to study the interaction with human leukocytes. SSL3 specifically interacted with human neutrophils (Fig. 1A) and monocytes (Fig. 1B), whereas almost no binding was observed for lymphocytes (Fig. 1C).

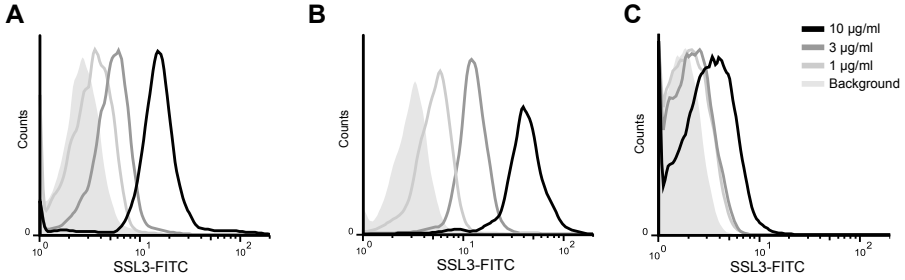


Figure 1. Binding of SSL3-FITC to leukocytes
Leukocytes were incubated with 0, 1, 3 or 10 µg/ml FITC-labeled SSL3 for 30 min at 4°C. Neutrophils (A), monocytes (B), and lymphocytes (C) were gated according to forward- and side-scatter properties.

To identify the molecular target for SSL3 on phagocytes, we investigated whether SSL3 could block the binding of a large panel of monoclonal antibodies (mAb) recognizing 39 receptors, expressed on neutrophils and monocytes, with crucial functions in innate immunity (e.g. chemotaxis, activation, adhesion, and phagocytosis). We found that SSL3 specifically inhibited binding of the function-blocking TLR2 monoclonal antibody to neutrophils and monocytes

(Fig. 2A). Inhibition of other tested cell-surface receptors was not observed. The expression of TLR2 differed between cell-types; monocytes (Fig. 2B) expressed higher levels compared to neutrophils (Fig. 2C), whereas TLR2 was absent on lymphocytes (data not shown). SSL3 dose-dependently blocked binding of anti-TLR2 to monocytes (Fig. 2B) and neutrophils (Fig. 2C). The IC_{50} for monocytes was around 0.05 µg/ml SSL3 and for neutrophils around 0.02 µg/ml (Fig. 2D). This slightly lower half maximal inhibitory concentration corresponds with the lower expression of TLR2 on neutrophils. These data indicate that SSL3 efficiently blocks a domain of TLR2 that is important for its function.

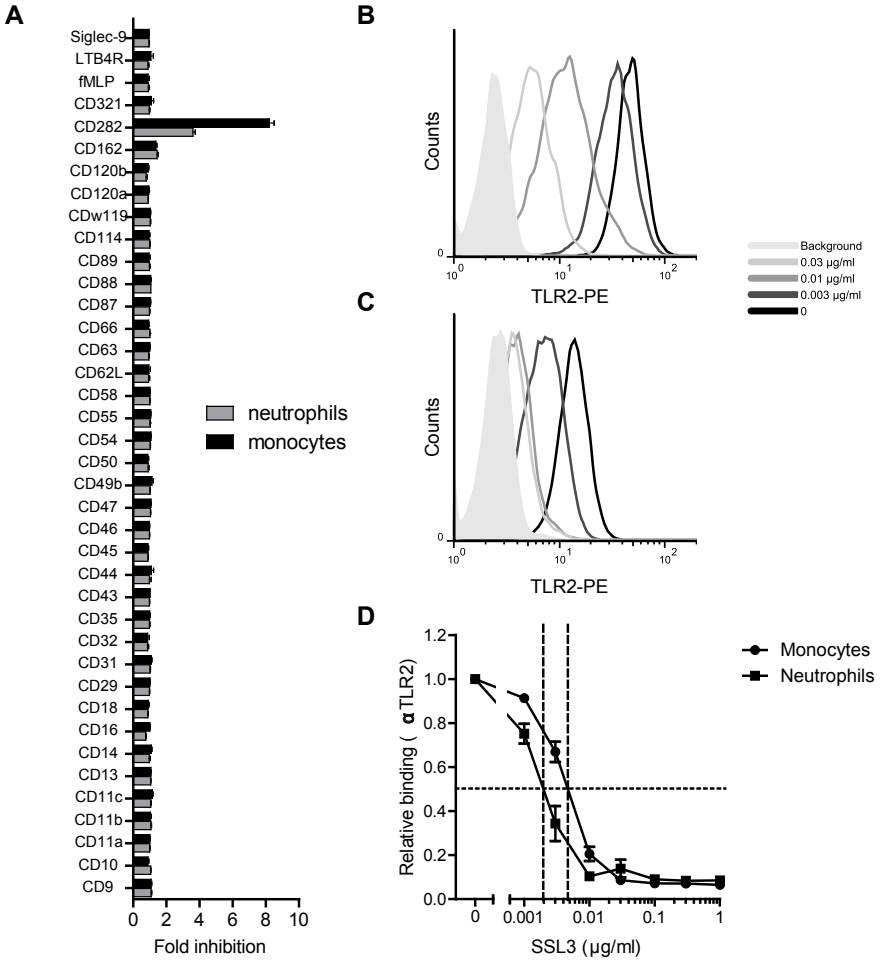


Figure 2. SSL3 competes with anti-TLR2 binding
(A) Leukocytes were preincubated with 10 µg/ml SSL3 for 30 min at 4°C, and subsequently incubated with a panel of different monoclonal antibodies directed against cell-surface receptors for 30 min at 4°C. Fold inhibition was calculated by dividing the fluorescence of untreated cells by that of treated cells. Data represent mean ± SEM of three independent experiments. (B-D) Leukocytes were incubated with various concentrations of SSL3 for 30 min at 4°C. Next, cells were incubated with PE-labeled anti-TLR2 for 30 min at 4°C. Histograms depict binding of TLR2 to neutrophils (B) and monocytes (C). Relative fluorescence (D) of anti-TLR2 binding to neutrophils and monocytes to calculate the IC_{50}. Data represent mean ± SEM of three independent experiments.

SSL3 inhibits TLR2 activation

To test whether SSL3, next to binding, could also inhibit TLR2 function, we stimulated HEK cells expressing TLR2 (HEK-TLR2) with the synthetic lipopeptides Pam2Cys and MALP-2, and measured the production of interleukin-8 (IL-8). SSL3 potently inhibited TLR2 activation by both agonists in a dose-dependent manner (Fig. 3A and 3B), confirming that SSL3 functionally inhibits TLR2. At 1 µg/ml SSL3, IL-8 production was abolished even when stimulated with 100 ng/ml Pam2Cys or MALP-2. Since TLR2 can dimerize with either TLR1 or TLR6 and thereby can discriminate between di- and tri-acylated lipoproteins and augment the cellular cytokine response, SSL3 inhibition was also tested on HEK-TLR2/6 or HEK-TLR1/2 cells activated with their specific synthetic ligands, MALP-2 (Fig. 3C) and Pam3Cys (Fig. 3D), respectively. SSL3 inhibited the IL-8 production of HEK-TLR1/2 cells, however inhibition was less potent in comparison with HEK-TLR2/6 cells.

The effect of SSL3 on TLR2 activation was also tested in primary human neutrophils and monocytes. In contrast to HEK-TLR2 cells, neutrophils and monocytes also express TLR4,

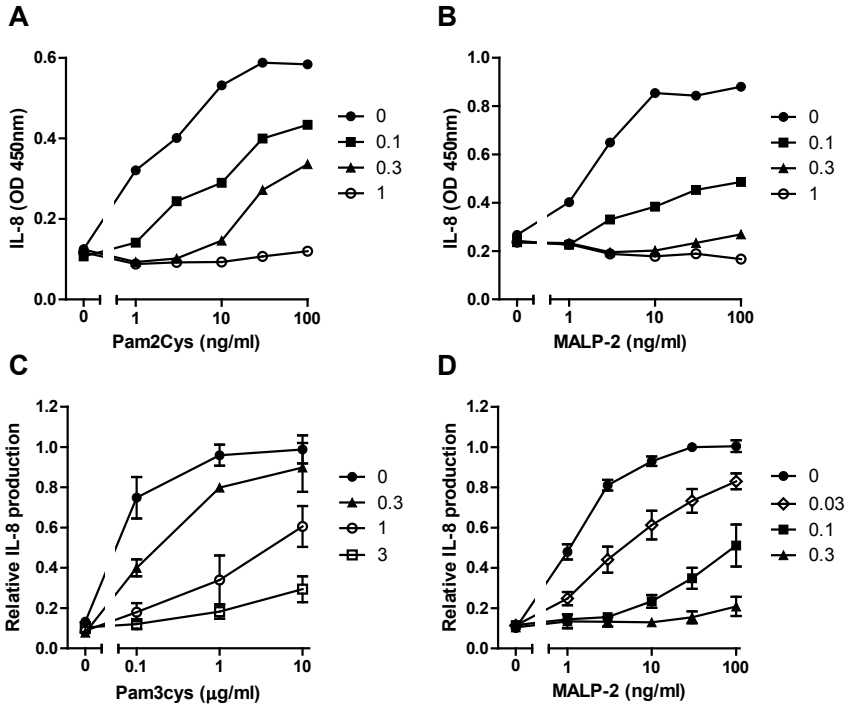


Figure 3. SSL3 inhibits TLR2 activation of HEK-TLR2 cells
(A,B) HEK cells transfected with TLR2 were incubated with 0, 0.1, 0.3 and 1 µg/ml SSL3 for 30 min. Cells were subsequently stimulated with increasing concentrations Pam2Cys (A) or MALP-2 (B). (C) HEK-TLR1/2 were preincubated with 0, 0.1, 1, and 10 µg/ml SSL3 for 30 min, and subsequently stimulated with various concentrations Pam3Cys. (D) HEK-TLR2/6 were preincubated with different concentrations SSL3 for 30 min, and subsequently stimulated with various concentrations MALP-2. All stimulations were performed overnight and cell supernatant was collected to measure produced IL-8 levels by ELISA. (A,B) IL-8 production is expressed as OD 450 nm. (C) The IL-8 production relative to cells stimulated with 1 µg/ml Pam3Cys was calculated and expressed as mean ± SD of triplicate experiments. (D) The IL-8 production relative to cells stimulated with 30 ng/ml MALP-2 was calculated and expressed as mean ± SEM of three independent experiments.

which can be activated in by lipopolysaccharide that is present in recombinant proteins generated in *E. coli.* To prevent IL-8 production via TLR4, we pretreated SSL3 with 20 µg/ml polymyxin-B to inactivate the lipopolysaccharide contamination. Additionally, PBMCs were pretreated with 10 µg/ml blocking anti-TLR4 mAb to prevent TLR4 activation. These precautions were sufficient to block TLR4 activation in both cell types, as even the highest concentration of SSL3, without addition of MALP-2, did not induce IL-8 production (Fig. 4A and 4B). In addition to HEK cells overexpressing TLR2, SSL3 also efficiently inhibited TLR2 activation by MALP-2 of both neutrophils (Fig. 4A) and PBMCs (Fig. 4B), as a source for monocytes.

SSL3 was not cytotoxic for cells, as verified by a lactate dehydrogenase (LDH) cytotoxicity assay performed on PMBCs and HEK-TLR2/6 cells after overnight incubation with SSL3 (Fig. 4C and 4D). SSL3 did not affect the IL-8 ELISA, as no difference in IL-8 standard curve was observed in the presence of 10 µg/ml SSL3 (data not shown).

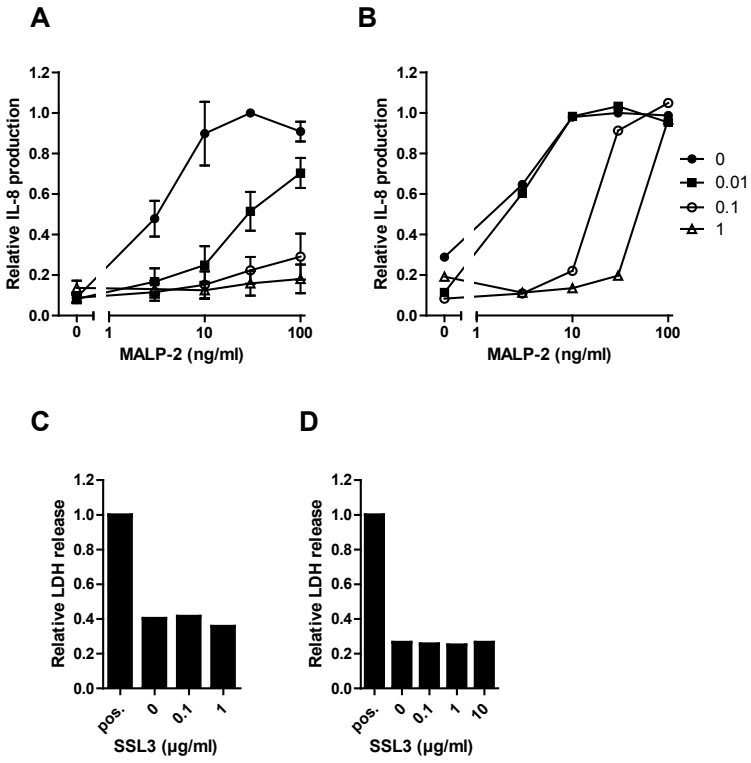


Figure 4. SSL3 prevents TLR2 activation of human leukocytes

(A,B) SSL3 was preincubated with 20 µg/ml polymyxin B and PBMCs were preincubated with 10 µg/ml anti-TLR4. Neutrophils (A) and PBMCs (B) were isolated from healthy donors and incubated with SSL3 for 30 min. Next, cells were stimulated with increasing concentrations of MALP-2. After overnight incubation, cell supernatant was harvested and IL-8 levels were determined by ELISA. Data are expressed as IL-8 production relative to stimulation with 30 ng/ml MALP-2. For neutrophils data represent mean ± SEM of three independent experiments and for PBMCs a representative experiment is shown. (C,D) Analysis of cytotoxic effects of SSL3 on PBMCs (C) and HEK-TLR2/6 cells (D). Cells were incubated overnight with SSL3 and toxicity was tested using the lactate dehydrogenase (LDH) cellular cytotoxicity detection kit. LDH is depicted relative to the positive control (lysed cells).

SSL3 recognizes both human and mouse TLR2

Thus far, the results strongly suggest that SSL3 is a specific TLR2 inhibitor. To further prove this, we investigated whether SSL3 binds to the extracellular domain of TLR2 since this domain is crucial for ligand recognition and TLR2 activation. Therefore, the extracellular domains of human and mouse TLR2, expressed in HEK293 cells, were purified and tested for binding to SSL3. ELISA studies showed that SSL3 effectively and dose-dependently bound to the extracellular domains of both human and mouse TLR2 (Fig. 5A). As SSL3 efficiently bound to human as well as mouse TLR2, we tested whether SSL3 could also inhibit the activation of TLR2 in the mouse macrophage cell line RAW264.7. Indeed, SSL3 also functionally inhibited mouse TLR2. SSL3 potently inhibited binding of the function-blocking anti–TLR2 to RAW264.7 cells (95.6 ± 0.95% inhibition at 0.1 μg/ml (data not shown). In addition, SSL3 completely blocked TLR2 activation by MALP-2, as measured by inhibition of TNFα production (Fig. 5B). Altogether we have shown that SSL3 is a specific and potent inhibitor of human and murine TLR2, which makes *in vivo* testing in mouse models feasible.

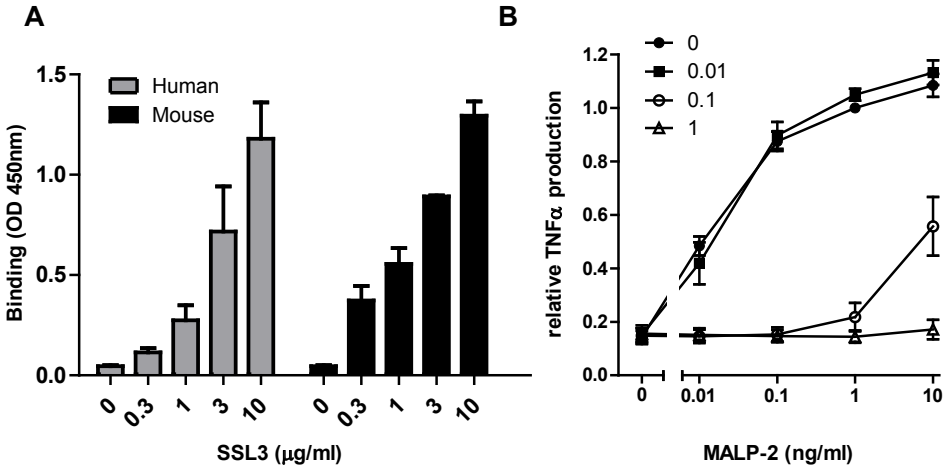


Figure 5. SSL3 binds and functionally inhibits mouse TLR2 activation
(A) A 96-wells plate was coated with the recombinant extracellular domain of mouse or human TLR2 (10 μg/ml). Coated wells were blocked with 4% skimmed milk, and subsequently increasing concentrations of His-SSL3 was added for 1 h at 37°C. Binding of SSL3 was detected with an anti-Xpress mAb, followed by a peroxidase-labeled goat anti-mouse IgG. (B) Mouse macrophage cells (RAW264.7) were preincubate with SSL3 for 30 min. Next, cells were stimulated with increasing concentrations MALP-2. After overnight incubation, cell supernatant was collected and TNFα levels were determined by ELISA. Data are expressed as TNFα production relative to cells stimulated with 1 ng/ml MALP-2 and represent the mean ± SEM of three independent experiments.

SSL3 targets exclusively TLR2

TLRs, including TLR5, induce intracellular signalling via the common adaptor protein MyD88. To exclude an effect of SSL3 on this common TLR signalling pathway, we tested whether SSL3 could inhibit TLR5 activation. Therefore, HEK-TLR5 cells were activated with flagellin, a TLR5-specific ligand. SSL3 could not inhibit flagellin-induced IL-8 production of neutrophils (Fig. 6). In contrast, AprA, which degrades flagellin and thereby prevents TLR5 activation (Chapter 2), abolished flagellin mediated IL-8 production (Fig. 6). Polymyxin B was added to prevent TLR4 dependent IL-8 production as a result of endotoxin contamination of SSL3. Addition

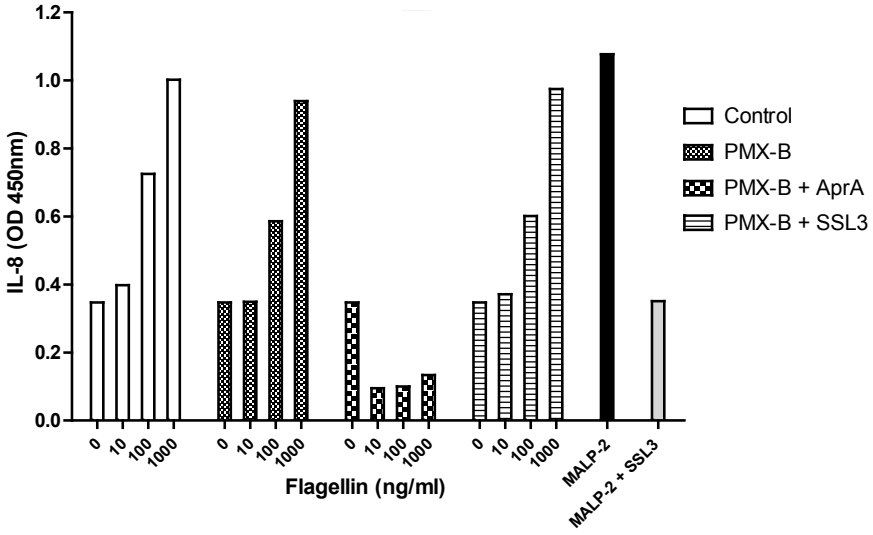


Figure 6. TLR5 activation is not inhibited by SSL3
Flagellin of *P. aeruginosa* was preincubated with polymyxin B (PMX-B; 20 µg/ml), PMX-B + AprA (10 µg/ml) or PMX-B + SSL3 (3 µg/ml) for 30 min at 37 °C. Neutrophils were stimulated overnight with treated flagellin at 37°C. In addition, neutrophils were stimulated with MALP-2 +/- SSL3 in the presence of PMX-B. Next, cell supernatant was collected and IL-8 production was measured by ELISA. Data are expressed as absorbance at 450 nm.

of only Polymyxin B to flagellin did not change the flagellin-induced activation of TLR5. As control, IL-8 production by MALP-2 was inhibited by SSL3. These results exclude that SSL3 inhibits the common MyD88-mediated intracellular signalling cascade and specifically acts on TLR2.

Activity of other SSLs for TLR2

SSLs present in pathogenicity island SAPI2 share function and sequence homology. We tested whether these SSLs, SSL1 to 11, all from *S. aureus* strain NCTC 8325 could, as observed for SSL3, inhibit TLR2 activation. None of the other SSLs, except for SSL4, inhibited the MALP-2 induced IL-8 production by HEK-TLR2 cells using a concentration of 10 µg/ml (Fig. 7A). Amino sequence alignment of SSL3 and SSL4 showed that their C-terminal domain of about 200 amino acids show high homology, in contrast to the other SSLs, indicating that the TLR2-inhibiting activity resides in this region (Fig. 7B). Comparison of the C-terminal domains of *S. aureus* strains MRSA252 (SSL4-MRSA) and NCTC 8325 (SSL4-8325) revealed an amino acid sequence homology of 87% and 76 % with SSL3-8325, respectively (Fig. 7B). To check the TLR2 inhibiting activity of both SSL4 variants, we analyzed the effect of both proteins on HEK-TLR2/6 cells activated with MALP-2. SSL4-MRSA was about 10-fold more active then SSL4-8325, which correlates with the higher homology to SSL3 in the amino acid sequence alignment (Fig. 7C). However, SSL4-8325 (Fig. 7C) was still about 30-fold less active then SSL3-8325 (Fig. 3B). In conclusion, TLR2 inhibiting properties of SSL3 resides within its C-terminal domain.

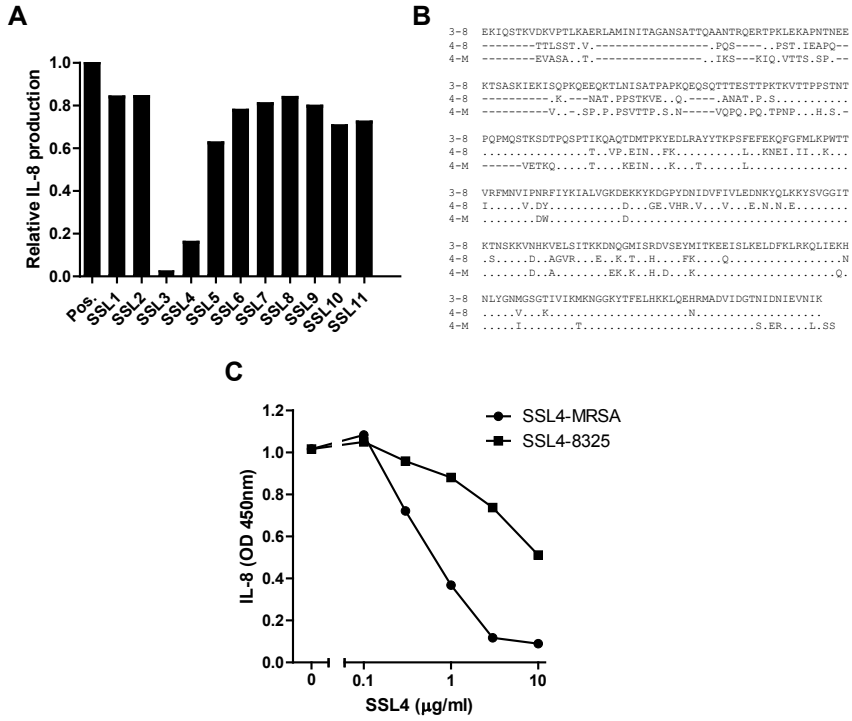


Figure 7 Effect of other SSLs on TLR2 activation
(A) HEK-TLR2/6 cells were preincubated with 10 µg/ml SSL1-11 for 30 min at 37°C, and subsequently stimulated with 3 ng/ml MALP-2. After overnight incubation, cell supernatant was harvested to determine IL-8 production by ELISA. IL-8 production is expressed relative to cells treated with MALP-2 only. (B) Homology of the amino acid sequences of SSL3 from *S. aureus* 8325 (3-8), SSL4 of 8325 (4-8) and SSL4 of *S. aureus* MRSA252 (4-M). (C) HEK-TLR2/6 cells were preincubated with increasing concentrations of SSL4-8325 and SSL4-MRSA252 for 30 min, and subsequently stimulated with 30 ng/ml MALP-2. After overnight incubation, cell supernatant was collected and IL-8 production was determined by ELISA. Data are expressed as absorbance at 450 nm.

Discussion

TLR2 has been shown to be critical for host protection against several bacterial infections including *S. aureus* [17,18,19,20,21]. To our knowledge, SSL3 is the first bacterial protein that specifically binds and inhibits TLR2. Pathak et al. [34] described a direct interaction between the early secreted antigen ESAT-6 of *Mycobacterium tuberculosis* and TLR2. Binding of ESAT-6 to the extracellular domain of TLR2 activated the intracellular signalling molecule Akt and prevented interaction between adaptor MyD88 and downstream kinase IRAK4. Therefore, ESAT-6 inhibited signalling by TLRs in general, not just by TLR2. We show that SSL3 cannot inhibit flagellin-induced TLR5 activation, which excludes inhibition of a common downstream signalling pathway. In literature, other TLR2 inhibitors are described, however, they target downstream signaling pathways, but not TLR2 directly. For example, the small molecule compound E567 was described as a novel TLR2 signalling inhibitor. However, E567 inhibits not only TLR2, but also TLR4 activation, possibly by targeting the adapter proteins

MyD88 and MyD88 adapter-like, which are involved in both the TLR2 and TLR4 signalling pathways [35]. Furthermore, herpes simplex virus immediate-early ICP0 protein inhibited TLR2-dependent inflammatory responses and NF-κB signalling by degradation of TLR adaptor molecules, making it also not specific for TLR2 [36].

Several coreceptors are described to play a role in ligand-induced TLR2 activation. CD14 is described as a coreceptor not only for TLR4, but also for TLR2. The HEK-TLR cells used in the current study do not express CD14, but sCD14 present in the serum was sufficient for a proper TLR2 activation. Using neutrophils and PBMC that do express CD14, we confirmed the important role of CD14 for TLR2 signalling, as addition of 60bca, a CD14 blocking antibody completely abolished MALP-2 induced IL-8 production (data not shown). Also CD36 acts as a coreceptor for TLR2, initiating TLR2/6 signalling. The ectodomain of CD36 binds and transfers negatively charged diacylglycerol ligands to TLR2, in a CD14 dependent manner [37]. Others showed that the COOH-terminal domain of CD36 is required for binding and internalization of *S. aureus*, which activates TLR2/6 signalling. As a result, CD36 deficient mice failed to efficiently clear *S. aureus* resulting in profound bacteremia [38]. TLR2-mediated activation of human monocytes by lipoproteins was also shown to require vitronectin and its receptor integrin β_3, as Glanzmann thrombasthenia patients, which lack integrin β_3, were unresponsive to lipoproteins [39].

It is unlikely that inhibition of TLR2 activation by SSL3 involves the interaction of SSL3 with one or more of the above described TLR2 coreceptors, as we clearly show cell independent binding of SSL3 to the recombinant extracellular domains of human and mouse TLR2. SSL3 probably competes with ligand binding to TLR2. On the other hand, it might also interfere with TLR1/2 or TLR2/6 dimerization, which is essential for TLR2 signalling [12]. We show that SSL3 inhibits binding of anti-TLR2 antibody T2.5 to cells expressing TLR2. This antibody was described to block ligand binding to TLR2 [40]. Moreover, anti-TLR2 antibody T2.5 was specific to both human and mouse TLR2, suggesting it to bind to the ligand specific epitope. These data suggest that SSL3 competes with ligand for TLR2 binding. The exact mechanism of how SSL3 inhibits TLR2 will be subject of future investigation.

All TLR2 inhibition experiments in this study were performed with SSL3 from *S. aureus* strain NCTC 8325 expressed in *E. coli*. A characteristic of the SSLs is that they display several short regions of high homology, which are necessary for their common structural folding. The same regions of high homology are found in the classical T cell superantigens, that display similar structures as SSLs [41]. These short regions of homology are not responsible for TLR2 inhibition, as none of the 11 SSLs were able to inhibit TLR2, except for SSL3 and 4. The TLR2 inhibitory activity of SSL4 *S. aureus* strain 8325 was 30 fold less as compared to SSL3. Sequence alignment of SSL3 and 4 suggests that the TLR2-inhibiting activity of SSL3 resides in its C-terminal 200 amino acids. This is underscored by SSL4 from *S. aureus* strain MRSA252 that is more homologous in its C-terminal domain to SSL3 as compared to SSL4-8325 and consequently displays a 10 fold higher TLR2 inhibition. The exact TLR2 binding site within SSL3 will be subject to future studies.

TLR2 is suggested to play an important role in many diseases, both infectious as noninfectious and both acute and chronic inflammatory diseases [42]. SSL3 might be helpful in the development of therapeutical agents to prevent or to treat some of these diseases. Structural studies with TLR2 in complex of SSL3 are necessary to reveal its active site, and will help to modify or to develop SSL3 derivatives that block TLR2 activation for therapeutic use. Since SSL3 also interacts and inhibits murine TLR2 these compounds can be readily

tested in mouse models.

SSL3 showed a high specificity for human neutrophils and monocytes and its inability to bind lymphocytes demonstrates its specificity for cells of the innate immune system. These cells pose the greatest threat for invading staphylococci. Two other cell-types involved in innate immunity, dendritic cells and macrophages express TLR2. As TLR2 on these cells plays an important role in the development of a proper adaptive immunity, SSL3 might in this way affect the adaptive immune response against *S. aureus*. Future studies with *S. aureus* SSL3 knockout strains in mouse infection models have to address the importance of SSL3 in *S. aureus* pathogenesis.

SSL3 is a unique TLR2 inhibitory protein and provides a powerful tool to study the thus far unknown exact molecular mechanism of TLR2 activation. Identification of the exact function and mechanism of TLR2 inhibition by SSL3 *in vitro* and *in vivo* will provide important new insights in the role of TLR2 in innate and adaptive immunity and in the pathophysiology of Staphylococcal infections and will aid in the design of future vaccines and anti-inflammatory therapies.

References

1. Barton GM (2007) Viral recognition by Toll-like receptors. Semin Immunol 19: 33-40.
2. Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373-384.
3. Chang ZL (2010) Important aspects of Toll-like receptors, ligands and their signaling pathways. Inflamm Res 59: 791-808.
4. Letiembre M, Echchannaoui H, Bachmann P, Ferracin F, Nieto C, et al. (2005) Toll-like receptor 2 deficiency delays pneumococcal phagocytosis and impairs oxidative killing by granulocytes. Infect Immun 73: 8397-8401.
5. Medzhitov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol 1: 135-145.
6. Ganley-Leal LM, Liu X, Wetzler LM (2006) Toll-like receptor 2-mediated human B cell differentiation. Clin Immunol 120: 272-284.
7. Iwasaki A, Medzhitov R (2004) Toll-like receptor control of the adaptive immune responses. Nat Immunol 5: 987-995.
8. Pulendran B (2004) Modulating vaccine responses with dendritic cells and Toll-like receptors. Immunol Rev 199: 227-250.
9. Booth J, Wilson H, Jimbo S, Mutwiri G (2010) Modulation of B cell responses by Toll-like receptors. Cell Tissue Res.
10. Zahringer U, Lindner B, Inamura S, Heine H, Alexander C (2008) TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology 213: 205-224.
11. Jin MS, Lee JO (2008) Structures of the toll-like receptor family and its ligand complexes. Immunity 29: 182-191.
12. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, et al. (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130: 1071-1082.
13. Schmaler M, Jann NJ, Gotz F, Landmann R (2010) Staphylococcal lipoproteins and their role in bacterial survival in mice. Int J Med Microbiol 300: 155-160.
14. Muhlradt PF, Kiess M, Meyer H, Sussmuth R, Jung G (1997) Isolation, structure elucidation, and synthesis of a macrophage stimulatory lipopeptide from Mycoplasma fermentans acting at picomolar concentration. J Exp Med 185: 1951-1958.
15. Schenk M, Belisle JT, Modlin RL (2009) TLR2 looks at lipoproteins. Immunity 31: 847-849.
16. Zahringer U, Lindner B, Inamura S, Heine H, Alexander C (2008) TLR2 - promiscuous or specific? A critical re-evaluation of a receptor expressing apparant broad specificity. Immunobiology 213: 205-224.
17. Archer KA, Roy CR (2006) MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of Legionella pneumophila in a mouse model of Legionnaires' disease. Infect Immun 74: 3325-3333.
18. Dickinson GS, Piccone H, Sun G, Lien E, Gatto L, et al. (2010) Toll-like receptor 2 deficiency results in impaired antibody responses and septic shock during Borrelia hermsii infection. Infect Immun 78: 4579-4588.
19. Mancuso G, Midiri A, Beninati C, Biondo C, Galbo R, et al. (2004) Dual role of TLR2 and myeloid differentiation factor 88 in a mouse model of invasive group B streptococcal disease. J Immunol 172: 6324-6329.
20. Takeuchi O, Hoshino K, Akira S (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol 165: 5392-5396.
21. Stenzel W, Soltek S, Sanchez-Ruiz M, Akira S, Miletic H, et al. (2008) Both TLR2 and TLR4 are required for the effective immune response in Staphylococcus aureus-induced experimental murine brain abscess. Am J Pathol 172: 132-145.
22. Bubeck Wardenburg J, Williams WA, Missiakas D (2006) Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins. Proc Natl Acad Sci U S A 103: 13831-13836.
23. Rooijakkers SH, van Kessel KP, van Strijp JA (2005) Staphylococcal innate immune evasion. Trends Microbiol 13: 596-601.

24. Lina G, Bohach GA, Nair SP, Hiramatsu K, Jouvin-Marche E, et al. (2004) Standard nomenclature for the superantigens expressed by Staphylococcus. J Infect Dis 189: 2334-2336.
25. Bestebroer J, Poppelier MJ, Ulfman LH, Lenting PJ, Denis CV, et al. (2007) Staphylococcal superantigen-like 5 binds PSGL-1 and inhibits P-selectin-mediated neutrophil rolling. Blood 109: 2936-2943.
26. Walenkamp AM, Bestebroer J, Boer IG, Kruizinga R, Verheul HM, et al. (2010) Staphylococcal SSL5 binding to human leukemia cells inhibits cell adhesion to endothelial cells and platelets. Cell Oncol 32: 1-10.
27. Bestebroer J, van Kessel KP, Azouagh H, Walenkamp AM, Boer IG, et al. (2009) Staphylococcal SSL5 inhibits leukocyte activation by chemokines and anaphylatoxins. Blood 113: 328-337.
28. de Haas CJ, Weeterings C, Vughs MM, de Groot PG, Van Strijp JA, et al. (2009) Staphylococcal superantigen-like 5 activates platelets and supports platelet adhesion under flow conditions, which involves glycoprotein Ibalpha and alpha IIb beta 3. J Thromb Haemost 7: 1867-1874.
29. Bestebroer J, Aerts PC, Rooijakkers SH, Pandey MK, Kohl J, et al. (2010) Functional basis for complement evasion by staphylococcal superantigen-like 7. Cell Microbiol 12: 1506-1516.
30. Walenkamp AM, Boer IG, Bestebroer J, Rozeveld D, Timmer-Bosscha H, et al. (2009) Staphylococcal superantigen-like 10 inhibits CXCL12-induced human tumor cell migration. Neoplasia 11: 333-344.
31. Itoh S, Hamada E, Kamoshida G, Yokoyama R, Takii T, et al. (2010) Staphylococcal superantigen-like protein 10 (SSL10) binds to human immunoglobulin G (IgG) and inhibits complement activation via the classical pathway. Mol Immunol 47: 932-938.
32. Patel D, Wines BD, Langley RJ, Fraser JD (2010) Specificity of staphylococcal superantigen-like protein 10 toward the human IgG1 Fc domain. J Immunol 184: 6283-6292.
33. Haas PJ, de Haas CJ, Kleibeuker W, Poppelier MJ, van Kessel KP, et al. (2004) N-terminal residues of the chemotaxis inhibitory protein of Staphylococcus aureus are essential for blocking formylated peptide receptor but not C5a receptor. J Immunol 173: 5704-5711.
34. Pathak SK, Basu S, Basu KK, Banerjee A, Pathak S, et al. (2007) Direct extracellular interaction between the early secreted antigen ESAT-6 of Mycobacterium tuberculosis and TLR2 inhibits TLR signaling in macrophages. Nat Immunol 8: 610-618.
35. Zhou S, Cerny AM, Bowen G, Chan M, Knipe DM, et al. Discovery of a novel TLR2 signaling inhibitor with anti-viral activity. Antiviral Res 87: 295-306.
36. van Lint AL, Murawski MR, Goodbody RE, Severa M, Fitzgerald KA, et al. (2010) Herpes simplex virus immediate-early ICP0 protein inhibits Toll-like receptor 2-dependent inflammatory responses and NF-kappaB signaling. J Virol 84: 10802-10811.
37. Jimenez-Dalmaroni MJ, Xiao N, Corper AL, Verdino P, Ainge GD, et al. (2009) Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2. PLoS One 4: e7411.
38. Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, et al. (2005) Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol 170: 477-485.
39. Gerold G, Ajaj KA, Bienert M, Laws HJ, Zychlinsky A, et al. (2008) A Toll-like receptor 2-integrin beta3 complex senses bacterial lipopeptides via vitronectin. Nat Immunol 9: 761-768.
40. Meng G, Rutz M, Schiemann M, Metzger J, Grabiec A, et al. (2004) Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. J Clin Invest 113: 1473-1481.
41. Fraser JD, Proft T (2008) The bacterial superantigen and superantigen-like proteins. Immunol Rev 225: 226-243.
42. O'Neill LA, Bryant CE, Doyle SL (2009) Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacol Rev 61: 177-197.

7

Summarizing Discussion

In this thesis, new bacterial immune modulators and new targets of already known proteases from *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been identified. These proteins interact and cleave various components of the innate immune system, and thereby they interfere with the host defense.

Immune evasion by bacterial proteases

Secreted bacterial proteases target a wide array of host proteins, such as antimicrobial peptides and components of the innate immune system [1]. These proteases range from very specific to broad specific proteases, which cleave a variety of different proteins. Proteases that have a high diversity of substrates tend to be nonspecific, and therefore the biological relevance of some in vitro substrates is questionable. In chapter 2, 3 and 5 functional experiments with alkaline protease and IMPa wer performed with concentrations in the range of the production in overnight bacterial culture supernatant. However, the production of pseudomonal proteases highly depends on culture conditions. Alkaline protease, IMPa (Chapter 2 and 5) and other pseudomonal proteases degrade multiple proteins involved in innate immunity. The advantage of secreting proteases for the bacterium is that they cleave different proteins, in comparison with small secreted immune evasion proteins, which bind to only one or two targets [2]. In addition, proteases can cleave many substrate molecules, whereas proteins that interfere via steric hindrance can only inactivate a limited number of molecules.

Several mammalian proteins are abundantly glycosylated, in contrast to bacterial proteins. By recognition of certain glycans, bacterial proteases can target host proteins dependent on their glycosylation status, and thereby discriminate between self and non-self proteins. The protease IMPa (Chapter 5) shows activity for the glycosylated cell-surface receptors CD43, CD44, CD55 and CD162 (PSGL-1). The activity of IMPa for CD43 and PSGL-1 depends on the cell-type that expresses these receptors, which is linked to the glycosylation status of both receptors. This suggests that IMPa activity depends on the recognition of certain glycosylation patterns. Although we show that the activity was not dependent on sialic acids, other glycan structures may determine protease specificity. The increased IMPa activity observed after removal of sialic acids for PSGL-1 and CD55 indicate that glycans also interfere with proteolytic activity. Other glycan structures that are present after sialic acid removal by neuraminidase can still be essential for protease activity and specificity. A protease of *Pasteurella haemolytica* also degrades CD43 and CD44, however activity of this protease, in contrast to IMPa, is dependent on sialic acids [3].

Protease inhibition by bacteria

The drawback of broad specific proteases is that they can also target bacterial proteins, and thereby inhibit growth or even kill bacteria. Some bacteria have developed specific inhibitors to inhibit their own protease. The alkaline protease inhibitor (AprI) of *P. aeruginosa* is a highly specific inhibitor of alkaline protease and is located in the periplasmic space, as predicted by its signal sequence (Chapter 4). Alkaline protease is normally secreted over both membranes by its own type I secretion system [4]. However, improper functioning of this secretion system may release proteolytic active alkaline protease in the periplasmic

space of the bacterium, resulting in degradation of bacterial proteins. The presence of AprI in the periplasmic neutralizes incorrectly translocated AprA, and thereby protects bacterial proteins against degradation. Alkaline protease inhibitor was not detected by specific polyclonal antibodies in bacterial lysates (data not shown), indicating that AprI is either present at low concentrations or expressed under different culture conditions. The presence of this specific inhibitor in all *Pseudomonas*, *Serratia* and *Erwinia* species, that secrete homologs of alkaline protease suggests that conservation of a functional AprI is essential for these bacteria.

Detection of bacteria triggers the degranulation of neutrophils, thereby releasing various proteases like neutrophil elastase and cathepsin G with antimicrobial activity. Furthermore, the activation of the complement system induces deposition of complement proteins with proteolytic activity on the bacterial surface. Therefore, inhibition of proteases at the bacterial surface is important to protect bacteria against phagocytosis, killing and degradation of bacterial surface proteins. *Streptococcus pyogenes* recruits protease inhibitors of the host to its surface [5]. The streptococcal surface protein GRAB binds alpha-2-macroglobulin (α2m), an abundant host plasma protein with broad protease inhibitory activity, and M protein recruits kininogens, which inhibit cysteine protease. Recruitment of broad specific protease inhibitors protect bacteria from host proteases. In addition, these protease inhibitors may protect bacteria against their own broad specific proteases, without affecting the function of these bacterial protease in degradation of host proteins and bacterial virulence.

Identification of Toll-like receptors modulators

Toll-like receptors recognize microbial components that are essential for their fitness. These structures range from flagellin, important for bacterial motility, to bacterial DNA. Some bacteria have managed to modify their conserved structures in such a way that they escape detection via TLRs, while functionality is maintained. Pathogens also interfere with TLR signaling via triggering of other cellular receptors [6,7]. The recent identification of secreted proteins that modulate the innate immune system in different bacteria [2,8] led to the hypothesis that bacteria also secrete proteins that inhibit TLR activation. We identified alkaline protease as an inhibitor of TLR5 (Chapter 2) and SSL3 as an inhibitor of TLR1, 2, and 6 signaling (Chapter 6). SSL3 of *S. aureus* is to our knowledge the first described bacterial inhibitor that directly interacts with a Toll-like receptor, and thereby sterically inhibits ligand binding.

The strategy to identify inhibitors in bacterial supernatant is quite straightforward when powerful functional screening assays are available, which are specific and not affected by other bacterial proteins like toxins. In addition, relative abundance in the secretome favors detection and easy purification of the protein of interest. The drawback of this strategy is that the production highly depends on culture conditions and that other secreted compounds interfere with target identification. For instance, the screening for TLR4 inhibitors was complicated (data not shown) due to the presence of high loads of lipopolysaccharide in the supernatant of Gram-negative bacteria. LPS potently triggers TLR4 activation of cells in biological assays and thereby impairs the detection of bacterial TLR4 inhibitors. Moreover, the complete removal of all LPS from proteins samples is complicated. Fishing with recombinant TLR4 in bacterial supernatant circumvents LPS contamination, however the expression and isolation of full-length extracellular TLRs is rather difficult. The structures of TLR2 and TLR4

in complex with their ligands were solved recently [9,10], which revealed the TLR domains involved in ligand binding. Expression and isolation of these smaller ligand binding domains is much easier and probably sufficient to identify interacting bacterial proteins in the future.

In vivo relevance of bacterial proteases

Bacterial proteases cleave host proteins with different efficiencies. Cleavage of purified proteins at high proteases concentrations can be biologically irrelevant, because the protease production in vivo is insufficient to reach the appropriate protease concentration for activity. In addition, protein-rich environments like in serum can inhibit broad specific proteases due to the fact that many other different substrates are present. At high concentrations, proteolytic activity of alkaline protease for complement C3 has been described [11]. However, we show in chapter 3 that C3 is not degraded by alkaline protease in serum. This indicates that alkaline protease prefers the cleavage of other substrates, which hampers the cleavage of C3. Analysis of other complement proteins revealed that alkaline protease degrades C2 and less efficiently C1s. Repletion experiments showed that only the cleavage of C2 is responsible for the inhibition of the classical and lectin pathway by alkaline protease of *P. aeruginosa*. The relatively low concentration of C2 in serum makes this component of the complement system an ideal target for proteolytic degradation.
Demonstration of protease activity in vivo is essential to conclude whether proteolytic degradation of substrates in vitro is biologically relevant. Alkaline protease degrades multiple proteins that have an important role in activation of the complement system (Chapter 3) and TLR5 recognition (Chapter 2). Therefore, animal models pinpointed to activation of complement or TLR5 will help to determine the effect of each pathway separately. Alkaline protease has been described as virulence factor in a *Drosophila* model [12]. Infection with a *Pseudomonas entomophila* mutant lacking the *aprA* gene resulted in a less persistent infection as compared to wild-type bacteria. This effect depends on the Toll pathway in *Drosophila*, which triggers the NF-κB transactivator Relish, as persistence of *aprA* knockout strains was restored in Relish deficient flies. Activation of the Relish pathway induces production of antimicrobial peptides, which are important in protection against invading pathogens. Flagellin triggers the production of antimicrobial peptides in flies [13], however no flagellin sensing receptor or homolog of TLR5 has been described in *Drosophila* yet. These results suggest that AprA may evade the production of antimicrobial peptides via the cleavage of monomeric flagellin in *Drosophila.* Studies in TLR5 knockout mice with *P. aeruginosa* lacking alkaline protease can further reveal the contribution of TLR5 evasion to *P. aeruginosa* virulence.

Therapeutic potential of proteases inhibitors

Small secreted proteins of *S. aureus* that target specific components of the innate immune system have therapeutic potential in inflammatory diseases [14]. In contrast to the relatively big and less specific pseudomonal proteases, IMPa and alkaline protease (Chapter 2 and 5), are not suitable for these kind of approaches. Inhibition of bacterial proteins that are involed in evasion of the innate immune system is a totally different strategy, which is described in chapter 4. Alkaline protease is associated with *Pseudomonas* virulence in

animal models and is detected in vivo in the sputa of cystic fibrosis patients [15]. Inhibition of alkaline protease will attenuate damage to host proteins and diminish pathogenicity of *P. aeruginosa* infection. The natural inhibitor of alkaline protease (AprI) occupies the active site of the protease with its N-terminus and thereby inhibit proteolytic activity. This activity is species specific as described in chapter 4. The inhibitor of *P. syringae* cannot interact and functionally block the alkaline protease of *P. aeruginosa* and vice versa. Six residues within the main body of AprI determines the species specificity. Introduction of these six residues of *P. aeruginosa* AprI in *P. Syringae* AprI results in a gain of function of this inhibitor. Understanding the enzyme-inhibitor interactions and inhibitor specificity is important to develop AprI derivatives that neutralizes alkaline protease and are suitable for therapeutic applications. A similar strategy has been exploited for *Pseudomonas* elastase, although the identified elastase inhibitor is a synthetic compound and not from bacterial origin such as AprI [16].

Therapeutic implication of SSL3 as anti-inflammatory compound

TLRs have an important function in several diseases including sepsis and asthma [17]. Several polymorphisms in human genes encoding TLRs or TLR signaling adapters have been associated with an increased risk of infections with different pathogens. Bacterial proteins like SSL3 that inhibit TLR activation have anti-inflammatory properties. SSL3 potently blocks activation of TLR2 by binding to the extracellular domain of both human and mouse TLR2. Therefore, the potency of SSL3 in vivo and the role of SSL3 in staphylococcal infections can be addressed in mice models. The use of bacterial proteins for therapeutic purposes is complicated, because these proteins induce antibody responses causing immune complex formation. However, characterization of the mechanism of inhibition can help in the development of therapeutic compounds or approaches to counteract TLR-mediated diseases.

Concluding remarks

P. aeruginosa and *S. aureus* secrete proteins that modulate Toll-like receptor activation by indirect (ligand degradation) and direct mechanisms (receptor binding or degradation of complement proteins). These proteins assist to escape detection by TLRs in combination with other bacterial strategies such as ligand manipulation and inhibition of intracellular TLR signaling. Along with the identification of TLR modulators, screening of bacterial supernatants revealed other pseudomonal proteins that display immune evasive features. The innate immune system detects and destroys bacteria by a wide variety of mechanisms. In response bacteria have evolved different strategies to survive within the host. They secrete proteins that interfere with all important strategies of the innate immune system to detect and eradicate pathogens. A wide array of these immunomodulators have been identified in different bacteria, however many uncharacterized secreted proteins remain that have a potential role in immune evasion.

References

1. Potempa J, Pike RN (2009) Corruption of innate immunity by bacterial proteases. JInnateImmun 1: 70-87.
2. Rooijakkers SH, van Kessel KP, van Strijp JA (2005) Staphylococcal innate immune evasion. Trends Microbiol 13: 596-601.
3. Abdullah KM, Udoh EA, Shewen PE, Mellors A (1992) A neutral glycoprotease of Pasteurella haemolytica A1 specifically cleaves O-sialoglycoproteins. Infect Immun 60: 56-62.
4. Tommassen J, Filloux A, Bally M, Murgier M, Lazdunski A (1992) Protein secretion in Pseudomonas aeruginosa. FEMS Microbiol Rev 9: 73-90.
5. Rasmussen M, Bjorck L (2002) Proteolysis and its regulation at the surface of Streptococcus pyogenes. Mol Microbiol 43: 537-544.
6. Hajishengallis G, Wang M, Liang S, Triantafilou M, Triantafilou K (2008) Pathogen induction of CXCR4/TLR2 cross-talk impairs host defense function. Proc Natl Acad Sci U S A 105: 13532-13537.
7. van Kooyk Y, Geijtenbeek TB (2003) DC-SIGN: escape mechanism for pathogens. Nat Rev Immunol 3: 697-709.
8. Laarman A, Milder F, van Strijp J, Rooijakkers S (2010) Complement inhibition by gram-positive pathogens: molecular mechanisms and therapeutic implications. J Mol Med 88: 115-120.
9. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, et al. (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell 130: 1071-1082.
10. Park BS, Song DH, Kim HM, Choi BS, Lee H, et al. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458: 1191-1195.
11. Hong YQ, Ghebrehiwet B (1992) Effect of Pseudomonas aeruginosa elastase and alkaline protease on serum complement and isolated components C1q and C3. Clin Immunol Immunopathol 62: 133-138.
12. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog 2: e56.
13. Samakovlis C, Asling B, Boman HG, Gateff E, Hultmark D (1992) In vitro induction of cecropin genes--an immune response in a Drosophila blood cell line. Biochem Biophys Res Commun 188: 1169-1175.
14. Laarman AJ, Ruyken M, Malone CL, van Strijp JA, Horswill AR, et al. (2011) Staphylococcus aureus Metalloprotease Aureolysin Cleaves Complement C3 To Mediate Immune Evasion. J Immunol.
15. Jaffar-Bandjee MC, Lazdunski A, Bally M, Carrere J, Chazalette JP, et al. (1995) Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by Pseudomonas aeruginosa. J Clin Microbiol 33: 924-929.
16. Cathcart GR, Quinn D, Greer B, Harriott P, Lynas JF, et al. (2011) Novel Inhibitors of the Pseudomonas aeruginosa Virulence Factor LasB: a Potential Therapeutic Approach for the Attenuation of Virulence Mechanisms in Pseudomonal Infection. Antimicrob Agents Chemother 55: 2670-2678.
17. Cook DN, Pisetsky DS, Schwartz DA (2004) Toll-like receptors in the pathogenesis of human disease. Nat Immunol 5: 975-979.

Nederlandse Samenvatting

Inleiding

De meeste bacteriën in ons lichaam hebben belangrijke functies zoals de vertering van voedsel en de ontwikkeling van het immuunsysteem. Sommige ziekmakende bacteriën (pathogenen) dringen door tot in de weefsels of bloedbaan en veroorzaken daar gevaarlijke infecties. Het immuunsysteem beschermt ons tegen indringers zoals bacteriën, maar ook virussen en schimmels, en kan verdeeld worden in een aangeboren en adaptieve tak. Het aangeboren immuunsysteem kan specifieke moleculen van bacteriën herkennen en roept de hulp in van onder andere neutrofielen uit het bloed, welke bacteriën kunnen opruimen (phagocytose). De detectie en vervolgens aanval om deze bacteriën te neutraliseren voltrekt zich in een kort tijdsbestek. Een belangrijk mechanisme om bacteriën te herkennen is via Toll-like receptoren (TLRs). Deze receptoren herkennen bacteriële moleculen die essentieel zijn voor de fitheid van de bacterie en vormen daardoor een ideaal doelwit voor het immuunsysteem. Doordat bacteriën altijd een aantal van deze moleculen bezitten is een select aantal receptoren in staat om alle bacteriën te herkennen. Detectie van deze microbiële structuren leidt tot activatie van cellen van het aangeboren immuunsysteem, waardoor de binnendringende bacteriën kunnen worden aangepakt. Het adaptieve (verworven) immuunsysteem reageert een stuk langzamer, maar ontwikkelt zich constant en onthoudt eerder gedetecteerde bacteriën om in het vervolg sneller en doelgerichter actie te kunnen ondernemen.
Pseudomonas aeruginosa (*P. aeruginosa*) is een bacterie die geen bedreiging vormt voor een immuunsysteem dat in goede conditie verkeert. In mensen met een verzwakte afweer kan deze bacterie echter serieuze infecties veroorzaken. Een voorbeeld hiervan zijn cystic fibrosis (taaislijmziekte) patiënten, deze hebben last van chronische *P. aeruginosa* luchtweginfecties die zeer slecht behandelbaar zijn met antibiotica. Om te overleven in de gastheer hebben bacteriën allerlei strategieën om herkenning door het aangeboren immuunsysteem te omzeilen. Voorbeelden hiervan zijn kapselvorming (beschermend omhulsel) en uitscheiden van eiwitten die cruciale functies van het aangeboren immuunsysteem blokkeren of componenten ervan afbreken met behulp van proteases. Deze proteases herkennen specifiek of minder specifiek eiwitten om deze vervolgens een of meerdere keren te knippen, zodat de functie van deze eiwitten verloren gaat. Het doel van het onderzoek dat beschreven is in dit proefschrift was het identificeren van nieuwe bacteriële eiwitten die de functie van Toll-like receptoren blokkeren.

Dit proefschrift

In **hoofdstuk 2** beschrijven we een nieuwe functie van een door *P. aeruginosa* uitgescheiden eiwit genaamd alkaline protease. Dit protease was al eerder beschreven als een virulentie factor van *P. aeruginosa* en knipt meerdere humane eiwitten. *P. aeruginosa* gebruikt een flagellum, een soort propeller, om zichzelf voort te bewegen. De bouwsteen van deze flagellum is flagellin, ongeveer 20,000 flagellin moleculen vormen een filament dat langer is dan de bacterie zelf. Cellen van het aangeboren immuunsysteem kunnen de aanwezigheid van bacteriën herkennen door flagellin te detecteren via een receptor op hun celoppervlak genaamd Toll-like receptor 5 (TLR5). Na herkenning van flagellin zorgt deze receptor voor activatie van cellen en het uitscheiden van stoffen (cytokines) die weer andere ontstekingscellen aantrekken naar de plek van infectie. Alleen monomeer flagellin en niet

flagellin in een intacte flagellum (polymeer, aaneenschakeling van monomeer flagellin) wordt herkend door TLR5. We hebben gevonden dat alkaline protease van *P. aeruginosa* het vrije (monomeer) TLR5-activerende flagellin afbreekt, en niet het flagellin dat aanwezig is in de flagellum (polymeer). Dit betekent dat *P. aeruginosa* niet zijn eigen flagellum afbreekt. Het afbreken hiervan zou namelijk erg ongunstig zijn voor de bacterie, omdat de flagellum essentieel is voor de beweeglijkheid van de bacterie. In feite ruimt *P. aeruginosa* zijn eigen flagellin op en voorkomt hiermee herkenning door het immuunsysteem via TLR5.
Alkaline protease van *P. aeruginosa* breekt meerdere eiwitten van het immuunsysteem af. We laten in **hoofdstuk 3** zien dat ook het complement eiwit C2 wordt afgebroken door dit protease. Het complement systeem bestaat uit 30 verschillende eiwitten die aanwezig zijn in het bloed en op verschillende manieren bacteriën herkennen en neutraliseren. Er zijn drie verschillende activatie routes van het complement systeem: de klassieke (herkenning antigeen-antilichaam complexen), de lectin (herkenning van suikers) en de alternatieve weg (amplificatie loop). Activatie van dit systeem leidt tot: labeling van de bacterie en vervolgens herkenning en afbraak door neutrofielen, uitscheiding van stoffen die neutrofielen naar de plek van infectie leiden en afhankelijk van de bacterie tot lysis (porie vorming in bacteriële membraan). C2 is in relatief lage concentraties aanwezig in het bloed, maar heeft een essentiële rol in activatie van het complement systeem. Hierdoor kan dit eiwit gezien worden als de achilleshiel van het complement systeem. Afbraak hiervan zorgt namelijk voor het grotendeels platleggen van dit systeem. Alkaline protease uitgeschieden door *P. aeruginosa* breekt C2 af en voorkomt daardoor activatie van de klassieke en lectin weg, welke beide afhankelijk zijn van C2. Hierdoor voorkomt *P. aeruginosa* de aantrekking en phagocytose door neutrofielen en lysis van de bacterie.
Zoals beschreven in hoofdstuk 2 en 3 is alkaline protease belangrijk voor *P. aeruginosa* om herkenning door het aangeboren immuunsysteem te ontwijken. Het remmen van dit protease zou dan ook een interessante mogelijkheid zijn om infecties met deze bacterie te bestrijden. *P. aeruginosa* maakt zelf een specifieke alkaline protease remmer (AprI) om zijn eigen eiwitten (binnen in de bacterie) te beschermen tegen afbraak door alkaline protease. In **hoofdstuk 4** hebben we deze natuurlijke remmer van *P. aeruginosa* gebruikt om te analyseren hoe dit protease geremd kan worden. We laten zien dat de inhibitor van *P. aeruginosa* alleen zijn eigen protease remt en niet het protease van een verwante bacterie, *Pseudomonas syringae* (*P. syringae*), en dus specifiek is. Aan de hand van de al bekende structuur van het protease-inhibitor complex hebben we mutaties aangebracht in AprI, om te kijken hoe deze inhibitor alkaline protease remt. Het introduceren van zes aminozuren van de *P. aeruginosa* remmer in de remmer van *P. syringae* zorgt ervoor dat er een functionele remmer ontstaat van *P. aeruginosa* alkaline protease. AprI is als eiwit minder geschikt voor therapeutische toepassingen, omdat toediening van een bacterieel eiwitten een ongewenste antilichaam respons opwekt. Het mechanisme van alkaline protease remming zorgt voor nieuwe inzichten om dit belangrijke protease van *Pseudomonas* en andere Gram-negatieve bacteriën te remmen en aan de ontwikkeling van therapeutische strategieën om *Pseudomonas* infecties te bestrijden.
P. aeruginosa scheidt vele immunomodulerende eiwitten uit. Een aantal van deze eiwitten zijn reeds geïdentificeerd, maar van veel uitgescheiden eiwitten is de functie nog onbekend. **In hoofdstuk 5** is beschreven hoe we tijdens het screenen van *P. aeruginosa* supernatant (vloeistof waar bacteriën in zijn gegroeid, na verwijdering van bacteriën) in verschillende immunologische assays op een eiwit gestuit zijn met een nog onbekende functie. Dit

protease hebben we immunomodulerend metalloprotease van *Pseudomonas aeruginosa* (IMPa) genoemd, omdat dit protease P-selectin glycoprotein ligand-1 (PSGL-1) en nog drie andere receptoren afbreekt die een rol hebben in celmigratie en adhesie. PSGL-1 speelt een cruciale rol in het aantrekken van cellen van het immuunsysteem uit het bloed naar de plek van infectie. IMPa breekt PSGL-1 af en voorkomt dat deze cellen naar de plek van infectie kunnen migreren.

In **hoofdstuk 6** beschrijven we de eerste bacteriële remmer van *Staphylococcus aureus* (*S. aureus*) die activatie van TLR2 remt via directe binding aan deze receptor. TLR2 herkent bacteriële modificaties van eiwitten die gebonden zijn aan de bacteriële membraan (lipoproteins). Deze eiwitten hebben verschillende functies en zijn belangrijk voor de fitheid van de bacterie, maar ze activeren tegelijkertijd TLR2. Verschillende eiwitten van de superantigen-like familie van *S. aureus* (SSLs) bezitten immunomodulerende eigenschappen. In deze studie hebben we SSL3, waarvan nog geen functie bekend was, in verschillende immunologische assays getest. Dit heeft geresulteerd in de ontdekking van SSL3 als TLR2 remmer. SSL3 bindt aan het extracellulaire domein van TLR2 en remt activatie van de receptor op neutrofielen, monocyten en cellijnen die deze receptor tot expressie brengen. Op deze manier voorkomt *S. aureus* herkenning en activatie van het immuunsysteem via TLR2.

Conclusie

In dit proefschrift hebben we nieuwe functies van uitgeschieden eiwitten van *P. aeruginosa* en *S. aureus* ontdekt. Deze eiwitten zorgen via verschillende mechanismen voor ontsnapping aan takken van het immuunsysteem. Naast deze en al eerder bekende eiwitten zullen er in de tokomst nog vele niet geïdentificeerde bacteriële eiwitten die vergelijkbare immunomodulerende functies hebben ontdekt worden. Het ontdekken van deze eiwitten en functies hiervan zijn belangrijk voor het begrijpen van *Pseudomonas* en stafylokokken infecties. Verdere studie naar de rol van deze eiwitten *in vivo* is belangrijk om te bepalen wat de relatieve bijdrage is in bacteriële infecties.

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Bart

Curriculum Vitae

Curriculum Vitae

Bart Bardoel was born on March 6, 1983 in Rotterdam, The Netherlands. He graduated from high school (VWO), Rotterdams Montessori Lyceum in 2001. In the same year he started his study Biomedical sciences at the University of Utrecht, and obtained his bachelor degree in 2004. During the master Immunity and Infection at the University of Utrecht, he performed his first internships under supervision of dr. E.F. Knol and dr. E. van Hoffen at the department of Dermatology/Allergology at the University Medical Center Utrecht. His second internship was under supervision of dr. S.H.M. Rooijakkers at the department of Medical Microbiology at the University Medical Center Utrecht, after which he graduated in 2006.
In September 2006, he started his PhD training at the department of Medical Microbiology at the University Medical Center Utrecht under supervision of prof.dr. J.A.G. van Strijp and dr. K.P.M. van Kessel. The results of this study are described in this thesis, and are published in different scientific journals.

Bart Bardoel
bartbardoel@gmail.com