Immune evasion and modulation mechanisms of *Pseudomonas aeruginosa.*

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Abstract

Pseudomonas aeruginosa is a Gram-negative bacterium, causing severe pulmonary infections in Cystic Fibrosis and immune-compromised patients. The immune responses induced by this bacterial infection and the chronic infection in the human host have been intensively studied in the past decades. More recently, attention has also been paid to immune evasion mechanisms of *P. aeruginosa*. Currently, a broad spectrum of immune evasion mechanisms of *P. aeruginosa* has been described, targeting different components of the immune system. In this review, an overview is given of the immune evasion and modulation mechanisms of *P. aeruginosa* in the human lung, but also in other hosts. In general, *P. aeruginosa* uses degradation of self-antigens, nonspecific proteases and exploitation of host immune regulators as main immune evasion strategies. The immune evasive components of *P. aeruginosa* contribute to bacterial proliferation and survival in the broad range of hosts of this pathogen and provide a source as antibiotic or vaccine targets for future applications.

Introduction

Pseudomonas aeruginosa is a Gram-negative pathogen that can cause severe disease upon colonization of the host. Although *P. aeruginosa* has a low morbidity and mortality in healthy individuals, immune-compromised and Cystic Fibrosis (CF) patients suffer from severe chronic pulmonary infections. In CF, 80% of the patients is colonized with *P. aeruginosa* at the age of 18, leading to decreased lung capacities and severe disease [1].

The *Pseudomonas* genus contains several pathogens which colonize many natural and cultivated environments and infect a broad range of hosts. One example is the major plant pathogen *Pseudomonas syringae* which affects over 50 cultivated plant species [2]. Although *P. aeruginosa* is independent of a host for efficient proliferation and survival, infection and colonization have been observed in plants (*Arabidopsis thaliana*), invertebrates (*Caenorhabitis elegans, Drosophila melanogaster*) and other mammals (*Mus musculus*) [3-5]. This efficient adaptability is partly due to the large genome of *P. aeruginosa*, containing 5.2 to 7 million base pairs and around 5500 open reading frames (ORF) [6].

How *P. aeruginosa* and other bacteria adapt to their host during infection and how they are able to chronically colonize human organs has been studied for years. From this perspective, special attention was paid to immune evasion mechanisms of the bacteria. With respect to *P. aeruginosa* host immune evasion, two major mechanisms were described for more than 20 years [7]. In his review, Kharazmi first describes the secretion of extracellular products by *P. aeruginosa* to escape host immunity. Secondly, *P. aeruginosa* was described to evade the immune system by biofilm growth during chronic infection, providing a barrier for proper immune function and antibiotic resistance [7]. More recently, immune evasion mechanisms of *P. aeruginosa* like quorum sensing, biofilm formation, flagellar motion and secreted proteases have been studied in more detail, leading to a better understanding of host-pathogen interactions and persistent infections in colonized patients [8, 9].

In this review, the current knowledge of host immune evasion by *P. aeruginosa* during pulmonary infections is summarized. First, the general characteristics of the host immune response for efficient eradication of *P. aeruginosa* pulmonary infections are described. Next, an overview is given of the current literature available addressing immune evasion mechanisms of *P. aeruginosa* during pulmonary infections in humans, but also in other hosts. Finally, *P. aeruginosa* immune evasion mechanisms are discussed from a broader perspective, addressing also implications for future research.

Immune responses against *P. aeruginosa*

Innate immune responses to PA

Pattern recognition

Recognition of Pathogen Associated Molecular Patterns (PAMPs) by Pattern Recognition Receptors is required for initiation of host immune responses during infection. In *P. aeruginosa* infections, flagellin and LPS are the major PAMPS, recognized by Toll like receptor (TLR) 5 and TLR4, respectively [9]. Although other TLRs like TLR2 and TLR9 also recognize *P. aeruginosa* patterns, they have an insignificant role during infections [9]. Upon TLR-recognition, Myd88-dependent signaling is induced, which is essential for clearance of pulmonary *P. aeruginosa* infections [10]. Myd88 signaling induces NFκB-dependent transcription of pro-inflammatory cytokines and chemokines, generating leukocyte activation and chemotaxis.

The crucial role for this signaling pathway during *P. aeruginosa* infections was illustrated by experiments using TLR4/5 double knockout mice [11, 12]. In these studies, impaired bacterial clearance and overall survival was observed upon *P. aeruginosa* infection. However, TLR4 or TLR5 -/- mice both eradicated *P. aeruginosa* infections completely, indicating that either TLR4 or TLR5 activation is sufficient for *P. aeruginosa* elimination.

The flagellar protein flagellin is the major ligand for TLR5 in mammals and FSL2 in plants [13, 14]. However, the epitope on *P. aeruginosa* flagellin required for TLR5 and FLS2 interaction is only free for binding in the monomeric form of flagellin, as this site is required for proto-filament formation and motility [13]. Monomeric flagellin is secreted by the bacteria, but, more important, is released upon intracellular lysis and killing [13]. Interestingly, flagellin was also shown to be secreted by non-motile, flagellum-expressing *P. aeruginosa* strains, inducing similar immune responses at very low concentrations [15]. During pulmonary *P. aeruginosa* infections, TLR4 and TLR5 signaling are essential in alveolar macrophages and epithelial cells for the production of inflammatory cytokines like IL-2 and chemotaxis of neutrophils to the site of infection [9].

A second important class of recognition receptors during *P. aeruginosa* infections is the Nod-like Receptor (NLR) family, inducing inflammasome activation and IL-1β production upon bacterial recognition. NLRC4 is the major NLR in *P. aeruginosa* infections, although the exact activation triggers are unclear until now. Some studies propose recognition of *P. aeruginosa* flagellin or motifs in the Type 3 secretion system (T3SS) by NLRC4, while others suggest that high extracellular potassium levels induce inflammasome activation [16, 17]. Stimulation of the inflammasome is crucial in airway epithelium during the acute stage of *P. aeruginosa* infection. Upon activation, secreted IL-1β activates the IL-1 receptor, inducing neutrophil recruitment to the site of infection [18].

The complement system

The complement system is an essential mechanism for bacterial clearance. Activation of the classical, lectin or alternative complement pathway occurs by recognition of antigen-antibody complexes, mannose-binding lectins or pathogen surfaces, respectively. The classical and lectin complement cascades are initiated by cleavage of C2 and C4, while factor B and D are crucial for activation of the alternative pathway. These different pathways lead to cleavage of the central component C3 into C3b and C3a. Activation of C3 induces cleavage of C5, resulting in the formation of the membrane attack complex (MAC) consisting of complement components C5b-9. [19]

The complement system fulfills multiple immune functions. First, complement activation leads to opsonization of the pathogen by C3b, enhancing phagocytosis and killing by neutrophils and other phagocytes. Second, release of complement components C3a and C5a induces inflammation and chemotaxis of innate immune cells to the site of infection. Finally, the membrane attack complex induces lysis of target cells, directly killing Gram-negative bacteria. [19]

In *P. aeruginosa* infections, C5a was shown to be essential for efficient phagocytosis and killing. In C5a-receptor deficient mice, bacterial eradication was strongly impaired, while normal recruitment of neutrophils

was observed [20]. In contrast, harmful effects of C3 for the host were reported by several studies, inducing severe neutrophil recruitment leading to chronic inflammation and tissue damage [9, 21].

As a consequence of the low specificity of the complement system, activation is strictly controlled. Passively, complement regulation is obtained by rapid inactivation of labile complement components. However, regulators of complement activation (RCAs) like complement receptor 1 (CR1), C4b-binding protein and factor H are also crucial for efficient control. Factor H is required for degradation of C3b upon activation by the alternative pathway and is exploited by *P. aeruginosa* to evade the complement system, which will be discussed later. [9, 22]

Phagocytosis and killing

Neutrophils are the major cells involved in phagocytosis and killing of pulmonary *P. aeruginosa* infections [9]. Upon pattern recognition of *P. aeruginosa* by alveolar macrophages and epithelial cells, chemokines like IL-8 are released which recruit the neutrophils to the site of infection. Neutrophils invade the tissue by selectin-mediated rolling, integrin-dependent adhesion to the vessel wall and chemokine-mediated transmigration and tissue-invasion. At the site of inflammation, neutrophils are activated by recognition of bacteria which are opsonized by complement component C3b, immunoglobulins (Ig) or surfactant proteins. Upon ingestion, the pathogen is killed in the phagolysosomes by proteolytic enzymes, reactive oxygen species (ROS) and nitric oxide. Moreover, the release of degrading enzymes upon neutrophilic lysis and the formation of neutrophil extracellular traps (NETs) were also shown to contribute to extracellular trapping and clearance of *P. aeruginosa* infection.[9]

However, in chronic pulmonary infections with *P. aeruginosa*, neutrophils also enhance tissue damage and inflammation. During apoptosis and phagocytosis, the release of proteases and NETs by neutrophils contributes to oxidative stress and increased inflammation. Clearance of apoptotic neutrophils and initiation of tissue repair occurs by alveolar macrophages, but is often insufficient during chronic infection in CF patients. [9]

Antimicrobial peptides

Extracellular, antimicrobial peptides (AMPs) like α-defensins, β-defensins and cathelicidins also contribute to neutralization and killing of *P. aeruginosa*. They are commonly produced by neutrophils and epithelial cells and secreted into the airway lumen. At high concentrations, AMPs kill microbes directly, often by membrane disruption. [23]

Adaptive immune responses to PA

Although clearance of *P. aeruginosa* strongly depends on the innate immune system, both the cellular and humoral parts of the adaptive immune response are involved during infection. However, the exact role of the adaptive immune system during *P. aeruginosa* infections is not completely understood.

The adaptive immune system is activated by antigen presenting cells (APCs) like dendritic cells (DCs) and macrophages. Upon phagocytosis, APCs present the microbial antigens to T-cells by Major Histocompatibility Complex (MHC) molecules. To induce T-cell maturation and proliferation, both MHC-presentation, co-receptor stimulation and cytokine production of the APC are required. These factors lead to T-cell differentiation, determining the direction of the adaptive immune response.

One direction is the cellular immune response, which is characterized by Th1 T-cells. Th1 T-cells produce high levels of cytokines, like IFNγ. These cytokines induce activation of IL-12 secreting macrophages and proliferation of cytotoxic T-cells, enhancing phagocytosis and killing of the pathogen by these cell types.

Humoral immunity is a second direction of the adaptive immune response. The major cells involved in the humoral immune response are B-cells and Th2 T-cells. By secretion of cytokines like IL-4 and IL-5, Th2 T-cells promote proliferation and activation of antibody-producing B-cells. The antibodies produced by B-cells opsonize the pathogen, inducing neutralization, complement activation and activation of neutrophils and other effector cells.

Upon *P. aeruginosa* infection, high antibody titers against different bacterial epitopes are induced, but these antibodies also have adverse effects for the host [24]. During chronic *P. aeruginosa* infections, the immune complexes formed upon opsonization also lead to tissue damage and chronic inflammation. Therefore, a harmful effect of the humoral response during *P. aeruginosa* infections has been suggested for years. [25]

The adverse effect of a dominant humoral immune responses during *P. aeruginosa* infections has been supported by other observations. First, comparisons between mice models showed that mice with a strong Th1 T-cell response had better outcome upon *P. aeruginosa* infections compared to mice with a dominant Th2 T-cell response [26]. Secondly, observational studies in chronically infected CF patients regularly demonstrated dominant Th2 T-cell responses, which are incapable of *P. aeruginosa* eradication [27]. However, the exact role of both Th1 and Th2 cells during pulmonary *P. aeruginosa* infections still has to be revealed.

Immune evasion by *Pseudomonas aeruginosa*

Biofilm formation and quorum sensing

Upon chronic infection in CF patients, *P. aeruginosa* changes from planktonic growth to a biofilm phenotype [28]. *P. aeruginosa* biofilms are often resistant against antibiotic treatment and, although both innate and adaptive immunity are activated, they are scarcely cleared by the immune system [25, 29, 30]. In CF, this bacterial immune resistance is the result of the complex interplay between host and pathogen in which impaired mucociliary clearance, immune defects and bacterial adaptations lead to persistent infection and chronic inflammation [30].

In the interactions between host and pathogen, several host immune factors contribute to bacterial survival during persistent infection. For example, proteolytic enzymes and reactive oxygen species have a dual role in bacterial clearance, as they not only destroy the bacteria, but also enhance impaired pathogen recognition by degradation of *P. aeruginosa* surface molecules [25]. In addition, the accumulation of extracellular DNA by the production of neutrophil extracellular traps (NETs) also has beneficial effects for bacterial survival. Extracellular DNA facilitates biofilm formation, enhances LPS modification by Mg^{2+} chelation and protects the bacteria from killing by antimicrobial peptides (AMPs) [31].

P. aeruginosa is also able to adapt to the human host during chronic infections. For example, by the secretion of proteases and down regulation of the bacterial flagellum, *P. aeruginosa* is able to evade the host immune system during chronic infections [25]. While these mechanisms are usually enhanced in *P. aeruginosa* biofilms, they are also observed in planktonic bacteria and therefore, they will be discussed in more detail below. Here, the immune evasive characteristics of the quorum sensing system are described, which enhance bacterial immune evasion in biofilms directly and indirectly [8].

Quorum sensing in *P. aeruginosa*

Quorum sensing is a density-dependent mechanism for interbacterial communication, based on secreted inducers, often called 'Quorum Sensing Signal Molecules' (QSSMs), which bind to a bacterial receptor. Upon inducer-receptor interaction, target gene transcription is induced, including transcription of the QSSM. In a growing population with high density, more inducer is synthesized. This causes a positive feedback loop, leading to full activation of the receptor and its target effects [32, 33].

In *P. aeruginosa*, three major QSSMs have been characterized, viz. the N-acyl-L-homoserine lactones N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL) and the Pseudomonas Quinolone Signal (PQS) [8, 34]. These QSSMs act, together with less dominant QSSMs, on the complex *P. aeruginosa* QS systems. The QS systems regulate large domains of the *P. aeruginosa* genome, enhancing biofilm maturation, swarming motility and secretion of many virulence factors [34]. Therefore, *P. aeruginosa* QS systems strongly contribute to bacterial virulence and immune resistance in plants, insects and mammals [35-37].

Immune modulating by *P. aeruginosa* QSSMs

QSSMs have a dual role during infection of *P. aeruginosa* biofilms, enhancing bacterial immune evasion directly and indirectly. Indirectly, QSSSMs are the main inducers of virulence during chronic infection, enhancing the secretion of immune evasive proteases and down regulating flagellar motility [34]. Direct effects of the QSSMs on the host immune system have also been described [8]. Here, the direct effects of 3-oxo-C12-HSL and PQS are discussed, as they are studied in more detail.

Primarily, 3-oxo-C12-HSL has been described to suppress Th1 and enhance Th2 T-cell responses *in vitro*. As described above, this means a decreased cellular response, by reduced production of the IFNγ and IL-12 and less proliferation of cytotoxic T-cells. In contrast, the humoral response is enhanced, inducing more production of IL-4 and IL5 and increased B-cell proliferation and antibody production.

In 1998, Telford *et al.* demonstrated that 3-oxo-C12-HSL, but not C4-HSL, inhibited proliferation of LPS-stimulated macrophages and production of TNFα and IL-12 [38]. Also, 3-oxo-C12-HSL dependent IL-4 production was observed in peripheral blood mononuclear cells (PBMC's) inducing IgE production. Similar observations were found by Chhabra *et al*, demonstrating down regulation of TNFα in LPS-stimulated leukocytes [39]. Also Hooi *et al* reported inhibition of cell proliferation in human leukocytes upon exposure of 3-oxo-C12-HSL and decreased IL-2 release [40]. The inhibitory effect of 3-oxo-C12-HSL on Th1 responses was supported by findings of Skindersoe *et al* [41]. In this study, 3-oxo-C12-HSL decreased IL-12 production in LPS-stimulated murine bone marrow-derived dendritic cells (BDDCs). These murine BDDCs reduced the induction of T-cell proliferation, leading to a decreased Th-1 response.

However, several others reported a rather a-specific effect of 3-oxo-C12-HSL on T-cell immunity. Ritchie *et al* demonstrated that stimulation with 3-oxo-C12-HSL inhibited the production of both IFN-γ and IL-4 in mice splenocytes and induced IgG1 production in OVA-immunized mice [42]. In this study, the authors suggest an a-specific effect of 3-oxo-C12-HSL on the immune system, modulating the existing T- and B-cell responses of the host [42]. Also in subsequent experiments, 3-oxo-C12-HDL was found to inhibit production of IFN-γ and IL-4 in $CD4^+$ T-cells [43]. Here, 3-oxo-C12-HDL was presumed to directly inhibit the initiation of T-cell activation, suppressing both Th1 and Th2 T-cell development.

Besides T-cell modulation, high dosages of 3-oxo-C12-HSL were also demonstrated to affect innate immune responses. In 2001, Smith *et al* showed that stimulation of human fibroblasts and epithelial cells with at least 100 μM 3-oxo-C12-HSL induced up regulation of NFκB and functional IL-8 [44]. In preceding experiment, NFκB-dependent up regulation of the inflammatory marker Cox-2 was observed in human lung fibroblasts [45]. However, for the dosages used in these studies, also cytotoxic effects have been reported [46]. Tateda *et al* demonstrated that stimulation with 25 μM and more 3-oxo-C12-HSL induces apoptosis in murine bone marrow derived macrophages (BDDMs), neutrophils and monocyte cell lines. Because of these findings, the immune effects reported for high dosages of 3-oxo-C12-HSL are questioned, promoting an immune-modulating effect of 3-oxo-C12-HSL rather than a pro-inflammatory effect on the host immune system [8, 41, 43].

A few studies indicate an effect of the pseudomonas quinolone signal (PQS) on the host immune system. Hooi *et al* reported a suppressive effect of PQS and 3-oxo-C12-HSL on T-cell proliferation. While 3-oxo-C12-HSL also inhibited IL-2 release, this was not observed for PQS, indicating a specific response of host cells to different QSSMs [40]. However, Skindersoe *et al* found no distinct effects of 3-oxo-C12-HSL and PQS on T-cell proliferation [41]. In this study, both 3-oxo-C12-HSL and PQS stimulation impaired IL-12 production in Bone Marrow-Derived Dendritic Cells (BMDCs), resulting in reduced T-cell proliferation *in vitro* [41].

All these data support the idea that both PQS and 3-oxo-C12-HSL modulate the host immune system during infection by down regulating the Th1 and probably also the Th2 T-cell response. Although the role of Th1 and Th2 T-cells during *P. aeruginosa* infections are not completely understood, Th2 T-cell responses are associated with impaired *P. aeruginosa* clearance [25-27]. Down regulation of the Th1 T-cell response fits in this model and could enhance overall survival and fitness of *P. aeruginosa* during infection. However, more studies are required to elucidate the exact role of both the Th1 and Th2 T-cell response during chronic *P. aeruginosa* infection and the effect of QSSMs on the Th1-Th2 balance.

Surface expressed molecules

Pseudomonas flagellin and the lipid A subunit of Lipo-poly-saccharide (LPS) are the major inducers for both animal and plant immune responses. In mammalian cells, LPS and flagellin are recognized by TLR4 and TLR5, respectively. In plants, flagellin is bound by FLS2 (flagellin sensing 2) while for LPS, no specific plant receptor has been described so far [47]. Upon TLR recognition in mammals, Myd88 signaling cascades are induced, leading to production of inflammatory cytokines and inflammation. In *P. aeruginosa* infections, either TLR4 or TLR5 signaling was shown to be sufficient for efficient induction of inflammatory responses [11]. Obviously, both flagellar motility and LPS based evasion mechanisms have been observed in different *P. aeruginosa* strains [48].

LPS

Differences in lipid A structures of *P. aeruginosa* LPS under different environmental conditions have been found for years. Environmental and laboratory *P. aeruginosa* strains PAO1 and PAK usually synthesize penta-acylated Lipid A, which barely induces a TLR4 response [49, 50]. In contrast, low magnesium culture conditions induce hexa-acylated Lipid A containing palmitate and aminorabinose. This hexa-acylated lipid A structure is associated with increased TLR4 signaling and inflammation [49, 51]. Similar Lipid A structures were found in early *P. aeruginosa* isolates from infected CF patients at the onset of infection [50]. Although several studies reported reduced magnesium levels in CF patients, the effect of this deficiency on *P. aeruginosa* Lipid A structures *in vivo* has not been elucidated until now [52, 53].

However, *P. aeruginosa* isolates from CF patients which were chronically infected for years, showed reduced TLR4 responses [54]. In contrast to early isolated strains, late strains induced less TLR4-dependent NFκB signaling and IL-8 production. Also, LPS isolated from early strains caused severe lesions and leukocyte recruitment in mice, which was strongly reduced in the late strains. Most likely, this differential inflammatory response between early and late CF strains is a result of differences in Lipid A acylation ratios. While late CF strains still contain hexa-acylated Lipid A, also under-acylated Lipid A isoforms are detected, which are associated with reduced cellular responses, indicating adaptation of the pathogen upon infection inside the host, driven by natural selection [51, 54].

Flagellar motility

As described above, flagellar motility of *P. aeruginosa* is also a major stimulus for the host immune system [14, 55, 56]. Still, one study reported an immune modulating effect of *P. aeruginosa* flagellin [57]. However, the contribution of these effects compared to the strong pro-inflammatory response induced upon TLR5 activation is unknown. Therefore, immune evasion of *P. aeruginosa* by impaired flagellar motility has been studied for many years. Decreased flagellar motility in clinical isolates of chronically infected CF patients has been reported for decades [58, 59]. Also, down regulation of the flagellum is a common phenomenon on *P. aeruginosa* biofilms, as it is supported by the quorum sensing system [34, 60]. However, how and to what extend loss of flagellar motility leads to increased immune resistance is still under debate.

Several studies suggest a TLR5-independent mechanism to evade the immune system by impaired flagellar motility [61-63]. In these experiments, *P. aeruginosa* (strain PA14) was able to evade non-opsonic phagocytosis by murine bone-marrow derived macrophages (BDMCs) and human peripheral blood mononuclear cells (PBMCs) by loss of motility, independent of flagellar expression [61, 62]. Interestingly, Myd88 knockdown had no effect on phagocytosis of both the motile and non-motile *P. aeruginosa* strains. More experiments demonstrated that inflammasome activation upon interaction of *P. aeruginosa* with phagocytes was also impaired by loss of bacterial motility and independent of flagellar expression [63]. These authors hypothesize a new immune recognition mechanism different from molecular patters, sensing bacterial locomotion.

In contrast with these findings, numerous studies highlighted the critical role of flagellar interactions with TLR5 for effective inflammasome activation, chemotaxis, phagocytosis and clearance of *P. aeruginosa* strain PAK [55, 64-66]. Also TLR5-mediated immune evasion was demonstrated to enhance bacterial survival

[67]. Interestingly, this is not only mediated by loss of flagellar motility, but also by degradation of monomeric flagellin which will be discussed in more detail below [67].

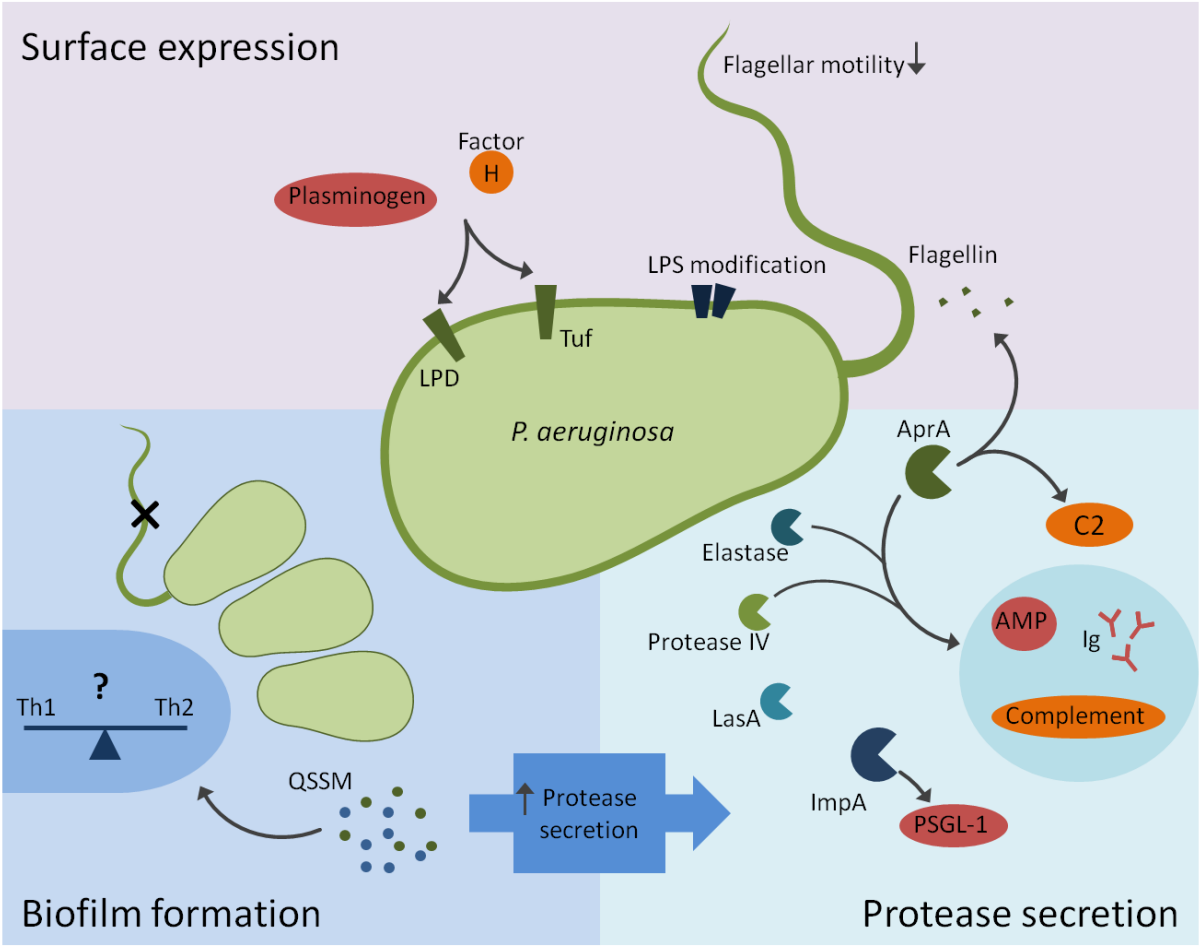

Figure 1: Overview of *P. aeruginosa* immune evasion mechanisms. *P. aeruginosa* immune evasion mechanisms are divided into three categories: biofilm formation and quorum sensing, surface expressed compounds, and secreted proteases, affecting host components (in red and orange) during pulmonary infections.

Tuf and Lpd

Several immune-evading characteristics have been described for elongation factor Tuf [68]. Elongation factors like Tuf are involved in translation and are commonly expressed intracellularly [69]. However, Tuf expression was also detected at the cell surface of at least 8 different *P. aeruginosa* clinical isolates. At the cell surface, Tuf is the first receptor of *P. aeruginosa* which was described to bind plasminogen. Plasminogen can be converted by host uPA into active plasmin, a key protease for degradation of extracellular matrix components. By degradation of extracellular matrix components, *P. aeruginosa* would hypothetically be able to invade the host tissue easier, but this has not been demonstrated so far [68]. Also, Tuf was described to bind complement regulator Factor H, which is still active upon interaction. By application of this host protease, *P. aeruginosa* uses acquired complement degradation for efficient evasion of the complement attack. Also in plants, and effect of Tuf was found. In *A. thaliana*, Tuf induces a plant defense response, leading to host resistance against bacterial infections [70].

Comparable properties were found for the recently characterized protein dihydrolipoamide dehydrogenase (Lpd). However, apart from the immune modulating capacities of Lpd, no other functions have been described until now. Like Tuf, Lpd is expressed at the cell surface of *P. aeruginosa* clinical isolates, although more variation is observed in expression quantities between individual clones [71]. At the cell surface,

Lpd contributes to bacterial survival in the host, as it binds plasminogen and complement regulator Factor H. These binding properties lead to degradation of the complement opsonin C3b, impairing neutrophilic phagocytosis and fibroblast growth. Next, binding of plasminogen leads to degradation of extracellular matrix, increasing invasion of the host tissue [71]. Although Lpd, and also Tuf, contribute to bacterial serum resistance of *P. aeruginosa in vitro,* the contribution of these factors to serum resistance compared to other endogenous and acquired complement evasion mechanisms of *P. aeruginosa* has not been fully elucidated.

Secreted products

Alkaline protease A

A major enzyme involved in *P. aeruginosa* immune evasion is the metalloprotease Alkaline protease (AprA). AprA is secreted by the type 3 secretion system and high levels have been reported in sputa of chronically infected CF patient [72-74]. Interestingly, AprA has been shown to degrade complement components and cytokines for years [75, 76]. An effect of AprA was also found in a *Drosophila melanogaster* infection model, where AprA protected *Pseudomonas* against antimicrobial peptides and contributed to persistent infections [77].

Recently, complement evasion by alkaline protease was studied in more detail by Laarman *et al* [78]. In this study, AprA was demonstrated to inhibit C3b deposition, C5a release and neutrophilic phagocytosis and killing. More specifically, AprA blocked the classical and lectin pathways by cleavage of C2, leading to increased bacterial resistance [78].

An additional target of AprA was reported by Bardoel *et al.* AprA cleaves monomeric, but not polymeric flagellin, inducing reduced mammalian TLR5 and plant FSL2 responses. *P. aeruginosa* containing mutant AprA, however, induced strong TLR5 and FSL2 responses in mammalian cells and plants, respectively. By degradation of monomeric flagellin, *P. aeruginosa* is able to evade immune recognition inside different hosts [67].

Elastase

Elastase is also a secreted protease of *P. aeruginosa.* Already in 1974, Schultz and Miller described the degradation of complement factors by elastase [79]. In addition, elastase was demonstrated to breakdown immunoglobulins, coagulation factors and the trypsin inhibitor alpha-proteinase [80]. Later, also fibroblast proteins and kinogens were established as degradation target of elastase and this broad spectrum of target protein still increases [81].

One study suggests an immune-modulating effect of elastase by degradation of surfactant protein A and D. As surfactant protein A and D are stimulating factors for both opsonic and non-opsonic phagocytosis by alveolar macrophages, degradation could result in impaired clearance of *P. aeruginosa,* while this has not been studied so far [82].

IMPa

Recently, IMPa was described as a new immune-modulating metalloprotease of *P. aeruginosa* [83]. Secreted IMPa cleaves PSGL-1, an important mediator in neutrophil recruitment. Functionally, IMPa treated neutrophils show impaired PSGL-1 mediated rolling, indicating a protective effect of IMPa against neutrophilic attack [83]. Protection from neutrophilic evasion by degradation of PSGL-1 has also been observed for *Staphylococcus aureus* secreted SSL5, but no IMPa related proteins have been observed in other *Pseudomonas* species [84].

Protease IV and LasA

Other secreted proteases, like Protease IV and LasA also contribute to bacterial virulence. However, their immune evasive properties are less studied so far [85, 86]. Some studies reported cleavage of complement factors, surfactant proteins and Immunoglobulins (Ig) by protease IV [85, 87]. Also some preliminary data indicate a role for Protease IV in complement degradation, but more experiments have to reveal the exact

targets and the immune-modulating properties of this protease during infection (Berends *et al,* unpublished data).

Discussion

Many opportunistic bacteria developed mechanisms to evade the host immune response, enhancing fitness and survival of the pathogen during infection [88, 89] . In recent years, more attention has been paid to bacterial immune evasion compounds as they are a potential drug target to eradicate the infection [78]. Additionally, the anti-inflammatory properties of these compounds make them also interesting in the search for immune-modulating drugs [78, 90, 91]. In this report, special attention was paid to *P. aeruginosa,* discussing the different immune evasion and immune modulating mechanisms of this pathogen during pulmonary infections as summarized in figure 1.

Although *P. aeruginosa* immune evasion has been studied for a long time, mechanisms like down regulation of flagellar motility, complement evasion and direct immune modulation by QSSMs are not fully elucidated yet. Partially, this is the result of missing experiments and conflicting data. Lacking experiments are found for example for the bacterial complement evasion mechanisms. Although complement regulation has been described for the *P. aeruginosa* proteins Tuf, Lpd, AprA, elastase and protease IV, it is less clear how the evasion molecules relate to each other. From this perspective, one study reported that exogenous complement evasion by the binding properties of Tuf was more potent than endogenous complement evasion by the protease activity of secreted AprA and elastase [68]. In addition, more efficient complement evasion was demonstrated for AprA compared to elastase [73]. These studies suggest that the distinct complement evasion components contribute differently to bacterial immune evasion *in vivo*, but this still has to be examined.

At first sight, conflicting literature is found for the more intensively studied immune modulating effects of *P. aeruginosa*, for example by QSSMs or by down regulation of flagellar motility [8]. However, a more detailed examination reveals major differences in experimental organization between studies. First, different bacterial strains are used, mainly PAO1 and PAK. These strains show large genotypical and phenotypical differences. Interestingly, PAO1 and PAK have distinct flagellar subtypes, although no differences in immune response have been observed between de different flagella until now [92, 93]. Second, more than 10^2-fold concentration differences are used for the *P. aeruginosa*-derived immune modulating components between different studies. The consequence of this difference is nicely illustrated in the QSSMs studies. Here, high concentrations gave different results compared to low dosages, but also had toxic effects [8, 44-46] Therefore, it is likely that the effects found for high concentrations are irrelevant during chronic pulmonary *P. aeruginosa* infections. Finally, conflicting results are often obtained by the use of distinct cell types from various hosts, impeding the comparability of the findings [8].

The effect of nearly all immune evasion mechanisms during pulmonary *P. aeruginosa* infections *in vivo* remains unclear until now. For almost all factors mentioned in this report, only *in vitro* experiments were performed, using planktonic *P. aeruginosa* laboratory strains. However, the extent to which these findings can be translated to clinical manifestations is questioned, which was nicely reviewed by Fux *et al* [94]. During chronic *P. aeruginosa* infections, the bacteria usually changes from planktonic growth to a biofilm phenotype [28]. Comparisons between laboratory planktonic *P. aeruginosa* strains and clinical isolates indicate that laboratory strains poorly represent the pathogenesis found during infection [94]. Therefore, *in vivo* experiments with different *P. aeruginosa* isolates are crucial to determine the relevance of *P. aeruginosa* immune evasion mechanisms during chronic pulmonary infection.

Although various mechanism are not examined to their full extend, three general immune evasion strategies of *P. aeruginosa* were elucidated in the described studies. As *P. aeruginosa* proliferation and survival is not restricted to the human host, a large proportion of these mechanisms also enhance survival in different hosts or by other mechanisms of action.

First, *P. aeruginosa* is able to adapt quickly to the host by degradation of self-antigens. This is illustrated by Lipid A modifications and down regulation of flagellar motion during infection, arresting TLR4 and TLR5 responses [50, 59]. Interestingly, the beneficial effect of *P. aeruginosa* adaptations is not restricted to the

human host, as breakdown of monomeric flagellin also contributes to bacterial survival in plants [67]. This degradation and down regulation of self-proteins is a method of *P. aeruginosa* to evade the immune system in different hosts and contributes to survival in different habitats.

Second, the main immune evasive secreted proteases of *P. aeruginosa* are non-specific, enhancing bacterial survival by several mechanisms. Secreted proteases like AprA, elastase and Protease IV have low specificity as they degrade both immune components, like the complement components, immunoglobulins and anti-microbial peptides, but have also other targets like host matrix components. By the use of multiple less specific proteases, *P. aeruginosa* targets the host immune response in different organisms at different levels, resulting in enhanced fitness in a broad host range. Because of the low specificity, it is unlikely that *P. aeruginosa* secreted compounds could be used as anti-inflammatory drug in future. However, several studies indicated that they can be used as vaccine or antibiotic targets to combat pulmonary *P. aeruginosa* infections in CF patients [95-97].

As a third strategy, *P. aeruginosa* uses host components for specific and efficient immune evasion. By adhesion of complement factors and plasminogen, *P. aeruginosa* exploits the anti-inflammatory mechanisms of the host. Beneficial effects of these mechanisms have not been described in other hosts so far, but are likely to be added in future.

From an evolutionary perspective, the described immune evasion mechanisms of *P. aeruginosa* suit well for a pathogen with a broad host range. By degradation of self-proteins, nonspecific disruption of host components and exploitation of host anti-inflammatory compounds, *P. aeruginosa* immune evasion mechanisms contribute to bacterial survival in different environments. This is in contrast with the immune evasion compounds of host-restricted pathogens like *S. aureus,* which specifically targets crucial human host immune compounds [98].

The broad spectrum of habitats of *P. aeruginosa* also raises the question to what extend host immune evasion is beneficial for the bacteria. Although colonized *P. aeruginosa* is highly adapted to the human CF lung by the formation of biofilms and LPS modification, the relevance of *P. aeruginosa* immune evasion mechanisms from a broader perspective is debatable. The opportunistic character of *P. aeruginosa* reveals that the healthy human lung is an unfavorable environment for efficient proliferation and survival. Moreover, *P. aeruginosa* survives often independent of a host, supporting the hypothesis that host immune evasion is not required for efficient proliferation.

Upon infection in the CF lung, *P. aeruginosa* is able to survive and exploits all the immune evasion mechanisms. However, although bacterial immune evasion mechanisms enhance bacterial survival, host immune defects also contribute to the persistent infections. Nowadays, it becomes more and more clear that many host and pathogen factors play a role during chronic *P. aeruginosa* infection, in which cause and effect are hard to distinguish.

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