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Stéphane Pont

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THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE GRENOBLE ALPES

Spécialité : **Virologie-Microbiologie-Immunologie**

Arrêté ministériel : 25 mai 2016

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préparée au sein du **Laboratoire Biologie du Cancer et de l'Infection**
dans l'**École Doctorale Chimie et Sciences du Vivant**

Strategies employed by *Pseudomonas aeruginosa* to withstand the innate immune system in human whole blood

Stratégies employées par *Pseudomonas aeruginosa* afin de résister au système immunitaire inné dans le sang humain

Thèse soutenue publiquement le **17 Novembre 2020**,
devant le jury composé de :

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Foreword

Pseudomonas aeruginosa is frequently isolated from the blood of bacteremic patients, and blood stream infections caused by this particular pathogen are associated with increased mortality rates compared to other bacterial species. Even though the interaction between *P. aeruginosa* and the major innate immune effectors of the circulation (i.e neutrophils and the complement system) have been investigated, these studies rarely assessed the behavior of this bacterium in human whole blood and rather used simplified *in vitro* models. As a consequence, we still lack the comprehension of the mechanisms that are used by *P. aeruginosa* to escape killing in the blood, and the information of which immune components are involved in the fight against this pathogen within the circulation.

The goal of my work was therefore to assess the behavior of *P. aeruginosa* in human whole blood, to better understand its interplay with the innate immune system in this particular context. The project aimed at deciphering the strategies employed by this bacterium to persist in the blood, and the essential immune components involved in the clearance of this pathogen. I also tried to identify new bacterial factors influencing the interaction between the bacteria and the complement system. This project started in 2016, when I performed my Master 1 internship under the supervision of François Cretin, who then gave me the opportunity to come back in the BPCR team for my Master 2 internship. Thanks to a PhD fellowship that I obtained from the French Ministry of Education and Research, I had the chance to spend three additional years in the team to complete this work.

The first section of the results chapter presents the main part of my project. Following an optimization step to overcome an unexpected concern in bacterial viability measurement, I assessed the survival of six *P. aeruginosa* strains in human whole blood and plasma. I identified that complement-resistance is the main driver of bacterial survival in this context, while the ability of the bacteria to destroy circulatory phagocytes does not predict the fate of the strain. I was also able to show that complement-sensitive strains of *P. aeruginosa* and some other Gram-negative human pathogens are able to prevent total clearance thanks to the presence of a subpopulation of “evaders”, tolerant to the lytic action of the complement but harboring a transient phenotype resembling antibiotic persisters. This project was performed partly in

collaboration with Laurence Van Melderen (Université Libre de Bruxelles, Belgium), which allowed to finalize and submit a first article.

In the second axis of my PhD, thanks to a genome-wide screen using a transposon mutant library (Tn-Seq), I identified new bacterial factors impacting the interaction between *P. aeruginosa* and the complement system in human plasma. This project is undergoing with Manon Janet-Maître, another PhD student of the team.

I was also involved in two other projects during these three years. In collaboration with clinicians from Paris and Besançon, I characterized the cytotoxicity of a hypervirulent strain of *P. aeruginosa* able to infect a healthy patient (Elabbadi et al., 2020). Finally, I had the opportunity to participate to a project led by Philippe Huber, researcher from the team, to assess the effect of inflammasome activation during lung infection with a specific strain of *P. aeruginosa* (Bouillot et al., 2020).

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List of acronyms

ADPRT: ADP-ribosyltransferase

AMPs: Antimicrobial Peptides

AP: Alternative Pathway

BALF: Bronchoalveolar Lavage Fluid

BSI: Bloodstream Infection

C4BP: C4 Binding Protein

CARD: Caspase Recruitment Domain

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

CFU: Colony Forming Unit

CLR: C-type Lectin Receptors

COPD: Chronic Obstructive Pulmonary Disease

CP: Classical Pathway

CR1: Complement Receptor 1

CRASPs: Complement Regulator-Acquiring Surface Proteins

CRs: Complement Receptors

DAF: Decay Acceleration Factor

DAMPs: Damaged Associated Molecular Patterns

DCs: Dendritic Cells

EPS: Exopolysaccharides

ETs: Extracellular Traps

ExlA: Exolysin A

ExlB: Exolysin B

FACS: Fluorescence-Activated Cell Sorting

FH: Factor H

FHL-1: Factor H-Like protein-1

FHR-1: Factor H-related protein-1

FI: Factor I

GAP: GTPase Activating Protein

GFP: Green Fluorescent Protein
GSDMD: Gasdermin D
HIP: Heat-Inactivated Plasma
ICUs: Intensive Care Units
IFN- γ : Interferon- γ
IL: Interleukin
IM: Inner Membrane
IRFs: Interferon Regulatory Factors
JAMs: Junctional Adhesion Molecules
LP: Lectin Pathway
LPS: Lipopolysaccharide
LRR: Leucine Rich Repeat
MAC: Membrane Attack Complex
MASPs: MBL-Associated Serine Proteases
MBL: Mannose-Binding Lectin
MCP1: Monocyte Chemoattractant Protein-1
MFI: Mean Fluorescent Intensity
MPO: Myeloperoxidase
NE: Neutrophil Elastase
NETs: Neutrophil Extracellular Traps
NF- κ B: Nuclear Factor- κ B
NLRs: Nod-Like Receptors
OM: Outer Membrane
OSA: O-specific antigen
PAMPs: Pathogen Associated Molecular Patterns
PASP: *P. aeruginosa* Small Protease
PCR: Polymerase Chain Reaction
PFTs: Pore-Forming Toxins
PG: Peptidoglycan
PMNs: Polymorphonuclear cells
PRRs: Pattern Recognition Receptors
PYD: Pyrin Domain

ROS: Reactive Oxygen Species

SCVs: Small Colony Variants

T3SS: Type 3 Secretion System

TLRs: Toll Like Receptors

Tn: Transposon

TNF- α : Tumor Necrosis Factor α

WT: Wild Type

Introduction

Chapter I

Bloodstream infections

1) State of the art

Bacteremia refers to the presence of viable bacteria within the normally sterile blood circulation. Asymptomatic bacteremia occurs regularly, as bacterial access to the bloodstream might be triggered by lesions like superficial wounds or minor bleedings during tooth cleaning (Lockhart et al., 2008). In these cases, commensal microorganisms from the skin or the oral microbiota enter the circulation. Non-compromised immune system is able to quickly eliminate non-pathogenic microorganisms from the blood, restoring the basal sterility without any complications (Lockhart et al., 2008). Unlike its asymptomatic counterpart, bloodstream infection (BSI) or septicemia, is one of the most serious life-threatening infection occurring when a bacterium enters the circulation and is not cleared by the immune system, either due to its pathogenicity or a weakness in the immune defenses. The incidence of such infection was recently evaluated as high as more than 150 cases per 100,000 population per year in Europe and North America, making it as prevalent as stroke (Kern and Rieg, 2020). Critically ill patients in intensive care units (ICUs) are particularly at risk, as shown by the fact that 15% of them were diagnosed with BSI (Vincent et al., 2009). In addition to their important frequency, BSI present an impressive mortality rate higher than 30%, which can reach 70% in the elderly, a population at risk for this kind of complication, in addition to neonates (Kang et al., 2003; Kern and Rieg, 2020; Leibovici-Weissman et al., 2019; Wójkowska-Mach et al., 2019). Septicemias represent also a burden in term of hospital charges, estimated at >\$40,000 per patient in the US (Kaye et al., 2014).

BSI occur *via* two main routes: either after the spread of a pathogen from a primary site of infection (i.e secondary BSI), with the urinary tract and the lungs being the major sources (Figure 1) (Bassetti et al., 2016), or through insertion of a contaminated needle or catheter directly into the circulation (i.e catheter-related septicemia), representing the primary cause of nosocomial bacteremia (Gahlot et al., 2014).

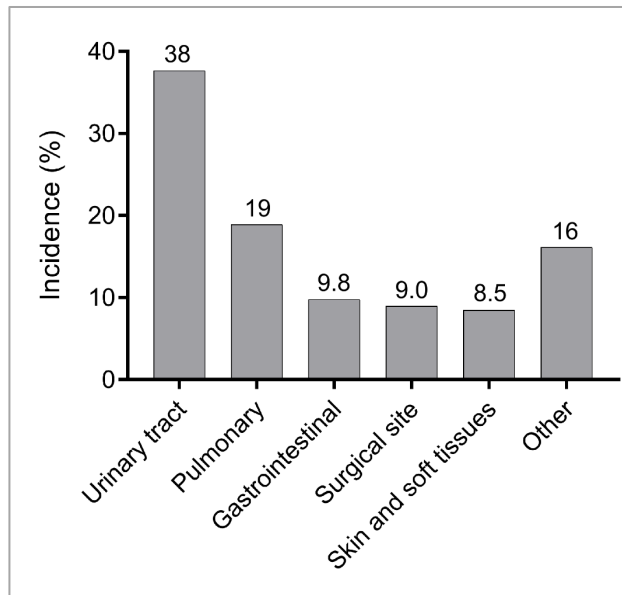


Figure 1: Primary sites of infection in secondary BSI. Data are from (Santé publique France 2019, data from the period May-June 2017).

Once in the vascular system, bacteria can disseminate within the body and affect organ functions depending on their intrinsic virulence and their ability to induce cell death. Meanwhile, the detection by the innate immune system of both the microorganism and any cell injury or tissue damages will induce the release of pro-inflammatory mediators (described in more details later) (van der Poll et al., 2017). The activation of the innate defenses is mediated through the interaction between pattern recognition receptors (PRRs), either soluble or at the surface of immune cells, and pathogen-associated molecular patterns (PAMPs) expressed by bacteria, or damage-associated molecular patterns (DAMPs, also known as alarmins) released by harmed eukaryotic cells. Along with the activation of the complement system in the blood (described in details later), generating the potent pro-inflammatory anaphylatoxins C3a and C5a, this immune response to foreign invaders can become out of control and may fail to return to steady state. This exacerbated inflammatory reaction is the major cause of sepsis, a complication associated with increased body temperature, heart and respiratory rates (van der Poll et al., 2017). Several international conferences attempted to define a consensus around the definition of sepsis (Bone et al., 1992; Levy et al., 2003; Singer et al., 2016), which finally was described as a ‘life-threatening organ dysfunction caused by a dysregulated host response to infection’, meaning that sepsis is not restricted to BSI. However, the presence of bacteria in the blood (i.e bacteremia) increases the mortality of septic patients (Komori et al., 2020). A

sustained inflammation increases the endothelial barrier permeability, resulting in septic shock characterized by a marked hypotension and multiple organ failure even after fluid perfusion, with a high mortality rate approaching 40% (Vincent et al., 2019). **Figure 2** recapitulates the evolution of a contamination toward BSI and septic shock.

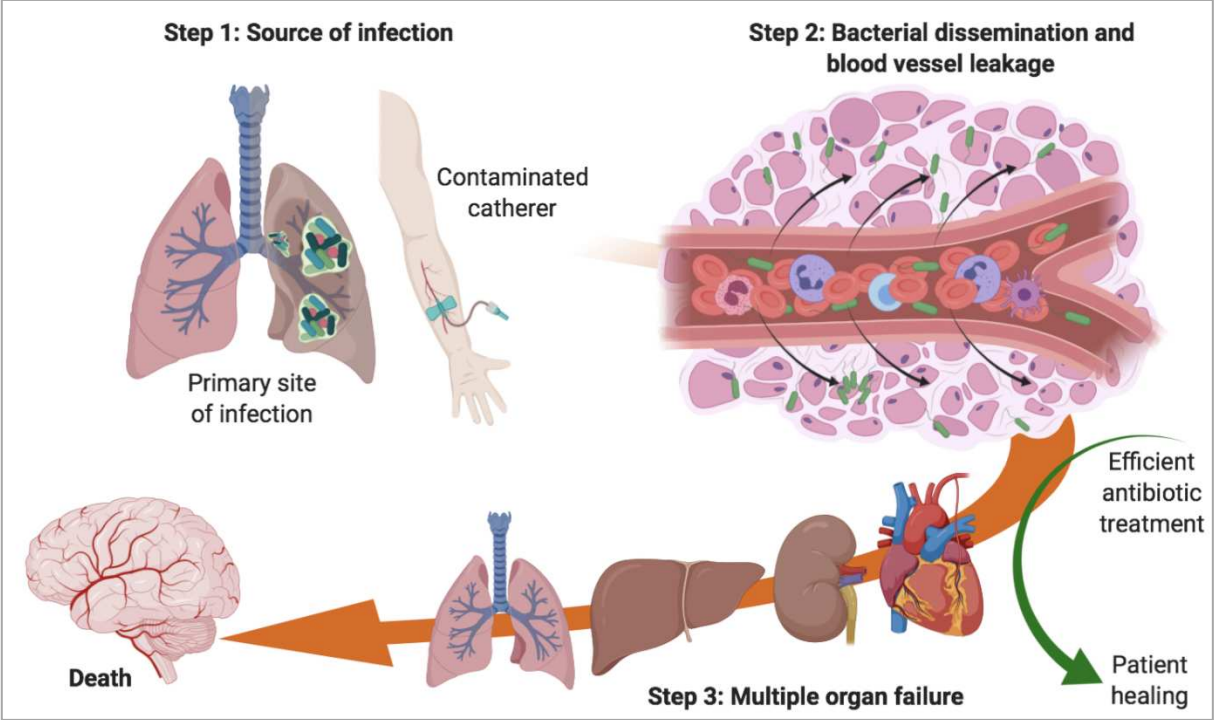


Figure 2: Major steps of a bloodstream infection. Either from a contaminated medical device such as catheter, or from a primary site of infection, bacteria reach the normally sterile circulation. Detection of the pathogen by the innate immune system induces a strong inflammatory response leading to endothelial barrier permeabilization, while the microorganism, if cytotoxic enough, disseminates in tissues distant from the initial site of contamination. Mainly due to the exacerbated pro-inflammatory reaction, hypotension deprives vital organs of essential components like oxygen and nutrients, potentially leading to death following septic shock. Chances of healing greatly increases if an efficient antibiotic treatment is administered to the patient.

BSI can be transient, intermittent or continuous, depending of their duration and if the status of bacteria in the blood alternates between presence and absence (Reimer et al., 1997). A plethora of bacterial species have been repeatedly isolated from the blood of patients suffering from BSI, with *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* species, *Pseudomonas aeruginosa*, enterococci, streptococci and coagulase-negative staphylococci being the most frequent (Figure 3) (Kern and Rieg, 2020; Reimer et al., 1997). Among these pathogens, *P. aeruginosa* bacteremias register with the highest mortality (Thaden et al., 2017). In addition to bacteria, the pathogenic yeast *Candida albicans* can also cause BSI, however its incidence is low in this type of complication.

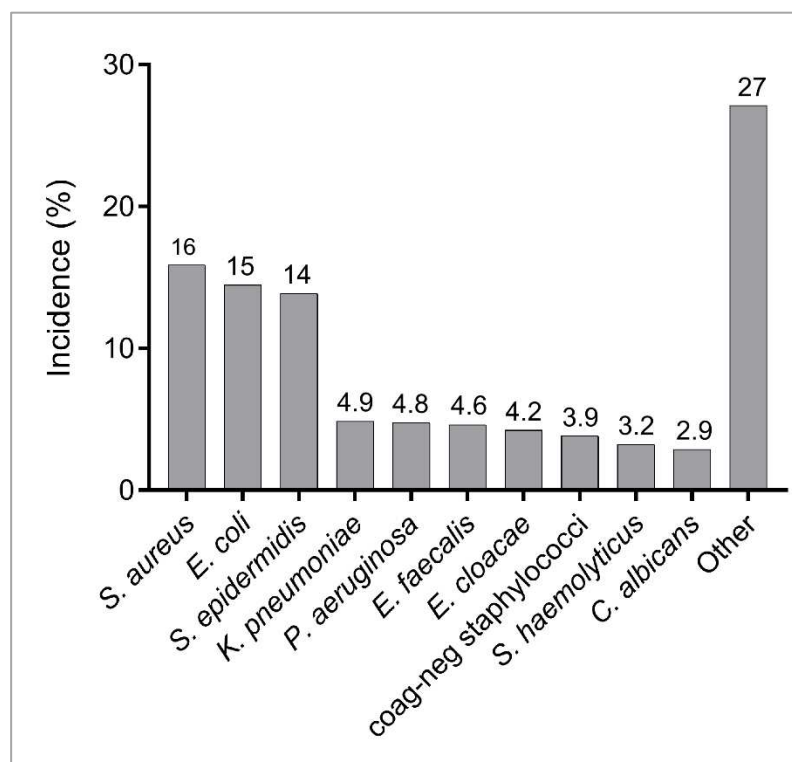


Figure 3: Major pathogens causing BSI. Data are from (Santé publique France 2019, data from the period May-June 2017). Coag-neg staphylococci: coagulase-negative staphylococci.

2) Requirements to reach the bloodstream from an infected organ

In the case of secondary BSI, bacteria are not primarily present in the circulation, and thus need first to withstand local innate immune defenses. Then, the pathogen has to cross the epithelial and endothelial barriers to finally reach the bloodstream, where it will face other

efficient immune effectors, which must be outsmarted for bacteria to disseminate through the organism. These different steps will be described, along with the strategies of various pathogens to reach that goal, with an emphasis on *P. aeruginosa* virulence.

a) Withstanding the local humoral innate immune defenses

The prevention of foreign invaders' entry within the organism is ensured by the innate immune defenses. This type of immunity is characterized by its rapidity of reaction (within minutes or a few hours) and its low specificity, meaning that it reacts in almost the same way regardless of the encountered pathogen. It is the exact opposite of the adaptive immunity, which is highly specific but takes time to implement (a few days), and confers a long-lasting protection thanks to the immunological memory, a process totally absent from its innate counterpart. The immune system is formed by two closely related arms, on one side the macromolecules forming the humoral immunity, and on the other hand the cells representing the cellular arm.

At pathogens' entry-sites, lysozyme is secreted by epithelial cells, and is thus found in most of our body secretions (tears, urine, saliva) and mucosal surfaces (Ragland and Criss, 2017). This enzyme with glycosidase activity exerts its antimicrobial function by disrupting the major component of the bacterial cell wall: the peptidoglycan (PG), a polymer of disaccharides. By breaking the β -1,4 bond between two PG monomers, lysozyme weakens PG integrity, subsequently rendering bacteria sensitive to osmotic pressure. Inherent to the structure of their membrane, Gram-positive bacteria are more easily targeted as their thick cell wall is in direct contact with the external environment, while in Gram-negative microorganisms the thin PG layer is sandwiched between the outer and the inner membrane, greatly reducing its accessibility (Figure 4).

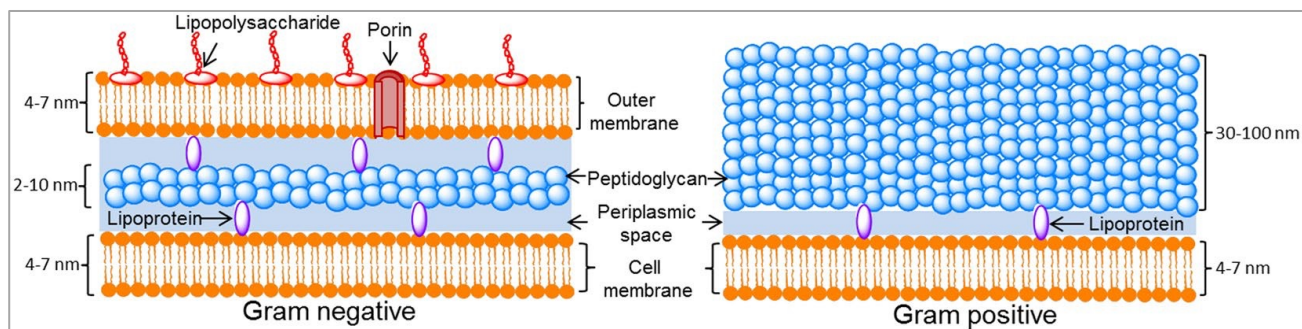


Figure 4: Structural differences between Gram-negative and Gram-positive bacterial membranes influence the action of lysozyme. Peptidoglycan is protected in Gram-negative microorganisms thanks to the presence of the outer membrane, physically preventing lysozyme to reach its target. In Gram-positive bacteria, PG is readily accessible for the protein to exert its antimicrobial enzymatic activity. From (Berezin et al., 2017).

To cope with this deadly effector, pathogens have developed different mechanisms, including PG modifications, production of lysosomal inhibitors and changes in surface charges (Ragland and Criss, 2017). Through specific *O*-acetylation, glycolylation or deacetylation of PG saccharides, bacterial species like *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Neisseria meningitidis* inhibit the binding of the enzyme to its target (Davis and Weiser, 2011; Ragland and Criss, 2017). Even though *P. aeruginosa* (among others) is unable to *O*-acetylate its PG, it can nonetheless resist lysozyme by producing specific inhibitors like the periplasmic proteins Ivy (inhibitor of vertebrate lysozyme) and MliC. However, they might exert their protective roles solely when the outer membrane is damaged by host's immune effectors (e.g antimicrobial peptides, bactericidal/permeability-increasing protein) making PG accessible (Abergel et al., 2007; Callewaert et al., 2008; Clarke et al., 2010; Deckers et al., 2008). By its cationic nature, lysozyme has also the ability to interact with and insert within the negatively-charged bacterial membrane, forming pores leading to cell lysis, without need of its enzymatic activity (Nash et al., 2006). By rendering their surface more neutral, microorganisms reduce their interaction with the antibacterial enzyme. This reduction of negative charges is achieved through biochemical alterations of membrane components, such as amidation of glutamic acid to glutamine in *S. aureus* (Figueiredo et al., 2012), modifications of the lipopolysaccharide (LPS) lipid A moiety through linkage of phosphoethanolamine in *A. baumannii* (Arroyo et al., 2011; Napier et al., 2013) or addition of aminoarabinose in *P. aeruginosa* (Moskowitz et al., 2004). These membrane modifications not only affect the interplay with lysozyme, but also participate to the pathogen's protection toward antimicrobial peptides (AMPs) (Ganz, 2003).

These small proteins (most of them <100 aa) produced by epithelial cells and neutrophils are found at high concentrations (>10 $\mu\text{g mL}^{-1}$) in some tissues and at epithelial surfaces. Two main families of AMPs have been identified: cathelicidins and defensins, differing in their structures and mode of activation (Izadpanah and Gallo, 2005). These innate immune effectors present an amphipathic structure, allowing them on one hand to bind the negatively charged bacterial surface through their cationic moiety, and insert within the membrane thanks to their hydrophobic patch, leading either to pore formation or lipid solubilization, causing the pathogen to lyse. Thus, by reducing the number of negative charges at their envelope, pathogens prevent the deadly interaction with AMPs. Proteases, like the elastase secreted by *P. aeruginosa*, have also been shown to degrade and inactivate the potent antimicrobial peptide cathelicidin LL-37 (Schmidtchen et al., 2002). Lysozyme and AMPs can act in concert to overcome pathogenic invaders, as illustrated by the concomitant action of lactoferrin, able to permeabilize the outer membrane of Gram-negative bacteria thanks to antimicrobial peptides found in its sequence (e.g lactoferricin), allowing the lysozyme to access the PG layer and exert its antimicrobial activity (Bruni et al., 2016; Ellison and Giehl, 1991) (Ellison and Giehl, 1991).

b) Preventing the induction of a proper inflammatory response

In addition to innate humoral immune macromolecules, peripheral tissues and mucosal surfaces also harbor resident immune cells, mainly dendritic cells (DCs) and macrophages. DCs form a subpopulation of sentinel leukocyte located at the pathogens' entry sites, which characteristics differ regarding their location within the organism. DCs are professional phagocytes able to engulf and kill pathogens. However, their role is not the direct clearance of invaders, but to activate the adaptive immune response specific to the encountered microorganism thanks to their antigen presenting capacities. Following pathogen sensing and phagocytosis in the inflamed tissues, DCs process antigens from the internalized microorganism and present them at their surface, bound to MHC class II molecules. During this process, DCs mature, allowing them to migrate to the closest draining lymph nodes. Within these secondary lymphoid organs and thanks to the antigens presented at their surface, DCs prime rare naive T cells specific for these antigens, initiating the induction of the adaptive immune response (Worbs et al., 2017). As these immune effectors are not primarily involved

in the direct fight against bacteria upon infection, their interaction with human pathogens will not be discussed. Macrophages are recognized as professional phagocytes, which role is to internalize and destroy any foreign invaders, in addition to alert other immune cells for the presence of pathogens. Depending of their location within the body, their properties can differ, giving rise to different subclasses like the alveolar macrophages in the lung and Kupffer cells in the liver (Laskin et al., 2001). To exert their sentinel role, macrophages express various PRRs, either membrane-bound or cytosolic, recognizing well-conserved microorganisms' structures not expressed by the host. Among these proteins, Toll-like receptors (TLRs) have been extensively studied and thirteen different members have been identified so far, each one recognizing different ligands (Figure 5). As an example, TLR4, in association with the two proteins MD2 and CD14, detects the LPS of Gram-negative bacteria, while TLR5 senses bacterial flagellin. Upon interaction with their specific ligand and thus bacterial recognition, TLRs dimerization induces a complex signalization cascade, eventually leading to the activation of the major transcriptional regulators nuclear factor- κ B (NF- κ B) and interferon regulatory factors (IRFs), depending on which pathogen's pattern is sensed (O'Neill et al., 2013). These transcription factors activate the expression of genes coding for chemokines and cytokines, which role is respectively to recruit and activate other immune cells where the pathogen enters. These molecules involved in autocrine, paracrine or even endocrine communication can be either pro-inflammatory, meaning that they promote activation and recruitment of other immune cells, or anti-inflammatory, when the immune response ends and the inflammation has to return to basal level following the resolution of the infection. Monocyte Chemoattractant Protein-1 (MCP1) and interleukin 8 (IL-8 or CXCR8) are two major chemokines released by epithelial cells and resident macrophages, the latter being the major source of these signaling molecules. Following the interaction with its target receptor CCR2 at the surface of monocytes, MCP1 recruits these phagocytic cells from the circulation, which transmigrate toward the infected tissues and differentiate in fully mature macrophages (Deshmane et al., 2009). On the other hand, the main function of IL-8 is to recruit high numbers of circulating neutrophils to the infection site. Indeed, upon IL-8 binding to its cognate G protein-coupled receptors CXCR1 and CXCR2 at the surface of polymorphonuclear cells (PMNs), intracellular Ca^{2+} concentration rises and SRC family kinases are activated. This signaling cascade induces the conversion of integrins into their high-affinity conformation, mediating neutrophil adhesion to the previously activated vascular endothelium located in the

vicinity of the pathogen's entry site (Nourshargh and Alon, 2014; de Oliveira et al., 2016; Zeilhofer and Schorr, 2000). Neutrophils then transmigrate from the luminal part of the capillaries toward the tissues, through a process known as diapedesis, during which PMNs cross the endothelial barrier either paracellularly (between cells) or transcellularly (through the cells), with paracellular migration being the main route used (~ 70% to 90% of the events). To that end, junctional proteins between endothelial cells maintaining vasculature integrity (e.g VE-cadherin and junctional adhesion molecules JAMs) have to be loosened. This process is achieved either through VE-cadherin endocytosis following its dephosphorylation (Sidibé and Imhof, 2014) or the degrading action of proteases released by neutrophils like the elastase (Hermant et al., 2003). Subsequent to diapedesis, PMNs follow the IL-8 gradient, which concentration increases along with the proximity to the infection site. Recruited neutrophils and monocytes eventually encounter the foreign invader and release signaling effectors, contributing to the proper development of the immune response. In addition to chemokines, pro-inflammatory cytokines released following NF- κ B activation are essential upon infection. The release of these molecules, among which TNF- α , IL-6, IL-1 β , IL-18 and IFN- γ are the most potent, activates immune cells present at the site of infection, increasing both their capacity to produce pro-inflammatory signals and their bactericidal activity (Nathan et al., 1983; Tan et al., 1995). Because a sustained inflammation can damage the tissues, this reaction needs to be stopped once the pathogen has been cleared. This process is achieved by the release of anti-inflammatory mediators like IL-10, which inhibit the production of pro-inflammatory effectors by target cells (Lyer and Cheng, 2012), and also by the fact that recruited neutrophils undergo apoptosis following microbial elimination. However, there is a growing body of evidence that some of these cells return to the bloodstream after bacteria have been destroyed (de Oliveira et al., 2016).

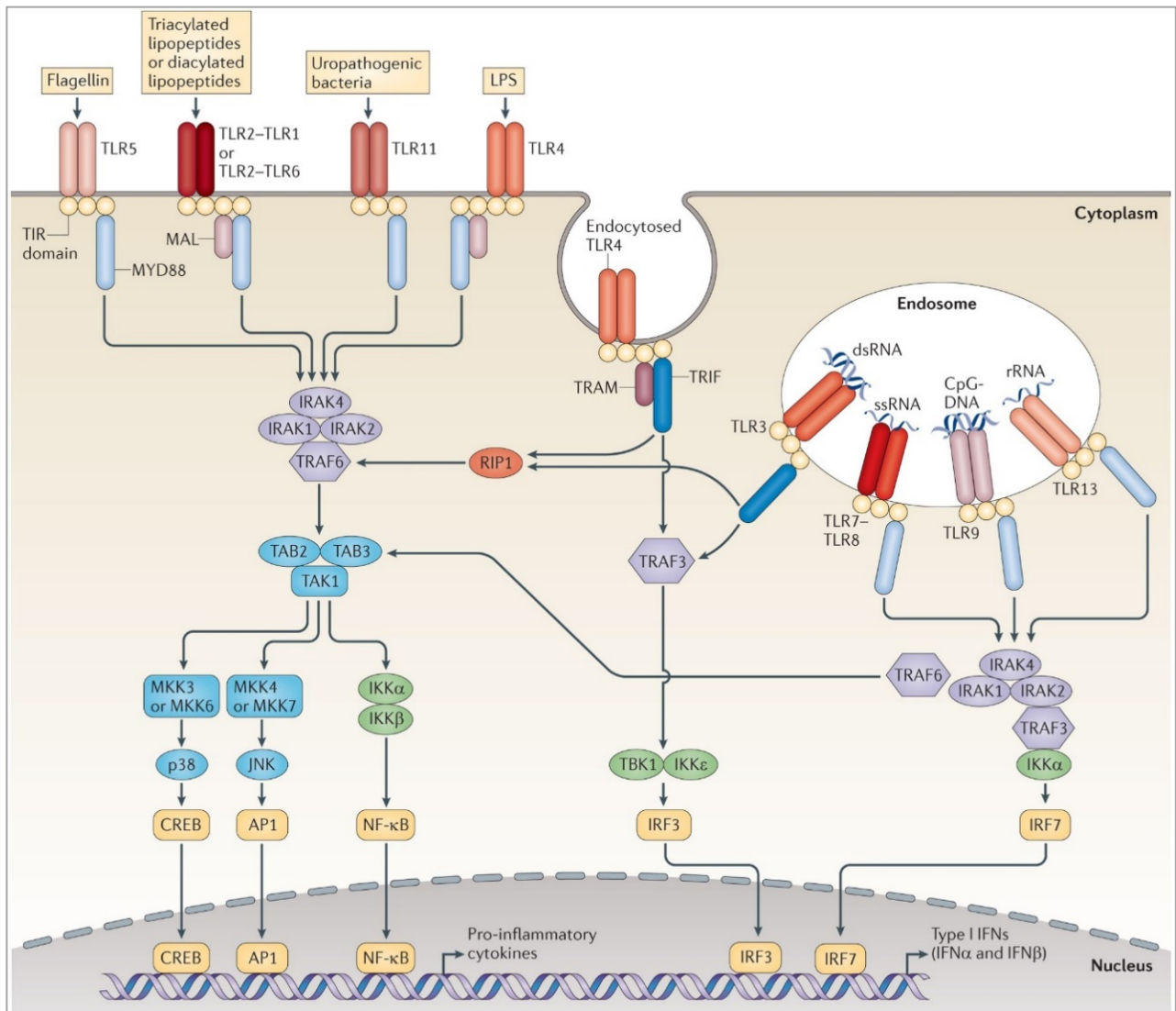


Figure 5: The wide variety of TLRs allows detection of a myriad of foreign macromolecules. Either exposed at the surface of the cell or lying within endosomes, TLRs detect specific ligands expressed by foreign microorganisms. Once activated, these receptors activate the transcription of pro-inflammatory cytokines genes, through the activation of NF- κ B and IRFs. From (O'Neill et al., 2013).

While TLRs allow the immune cells to detect harmful signals present in the extracellular and endosomal medium, nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are involved in the recognition of PAMPs and DAMPs found in the cell cytoplasm. The two major types of NLRs are the NLRC and NLRP proteins, with NLRC4 and NLRP3 receptors being the most studied one. They both carry a leucine rich repeat (LRR) and a NACHT domain, however members of the NLRC group present a caspase recruitment domain (CARD), while the NLRP lacks such motif and instead harbor a pyrin domain (PYD). Upon

LRR binding to its specific ligand, which nature varies between NLRs, the receptor is activated following conformational changes and interacts either directly (NLRC) or indirectly through the adaptor protein ASC (NLRP) with pro-caspase 1 (Figure 6) (Platnich and Muruve, 2019). Oligomerization of such complexes, through the ATPase activity of the NACHT domain, induces the formation of a multimeric platform known as the inflammasome. This structure allows neighboring pro-caspase 1 to activate each other following a proteolytic cleavage. Activated caspase 1 subsequently cleaves pro-IL-1 β and pro-IL-18, generating fully mature IL-1 β and IL-18 with very potent pro-inflammatory functions. As these cytokines do not harbor a signal peptide, their release within the extracellular medium is mediated through a programmed cell death known as pyroptosis, during which the protein gasdermin D (GSDMD) is activated by caspase-1 and subsequently oligomerize to form pores within the host's cell membrane, inducing cell's burst (Figure 7). By this fine-tuned programmed cell death, pro-inflammatory signals are released in the vicinity of the dying cells, contributing to the development of a proper inflammatory response. If the cell was infected with an intracellular pathogen, the microorganism becomes exposed to surrounding immune effectors activated by the release of mature IL-1 β and IL-18. TLRs signaling and NLRs activation are tightly linked as the transcription of genes coding for pro-IL-1 β and pro-IL-18 requires a primary induction after TLR detection of its cognate PAMP or DAMP, which can be signals released from pyroptotic cells (Figure 7). In the specific case of NLRP3, NLRs monomers are synthesized following a first priming signal dependent of TLR activation.

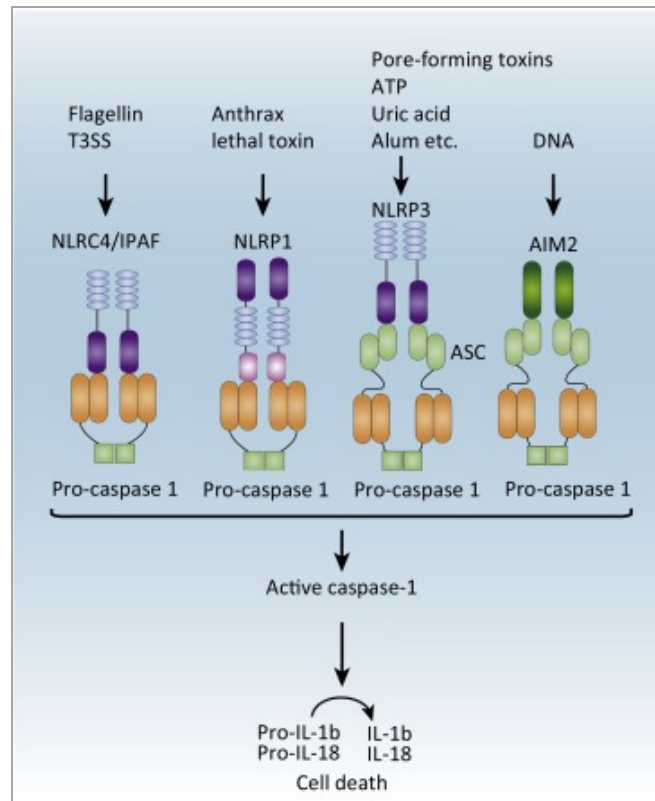


Figure 6: The most studied NLRs and their activating signals. Upon activation by their specific ligand, conformational changes trigger the activation of NLRs, which are then able to interact with pro-caspase 1, either directly or thanks to the adaptor protein ASC. Following caspase 1 activation, mature pro-inflammatory cytokines IL-1 β and IL-18 are generated. From (Vanaja et al., 2015).

Among the signals sensed by these intracellular PRRs, NLRC4 detects bacterial proteins directly injected within the cytoplasm of the cell. Indeed, upon infection, certain Gram-negative bacteria like *P. aeruginosa* or the intracellular pathogen *Salmonella enterica* use their type 3 secretion system (T3SS), a complex injectisome present at their surface, to deliver toxic effectors within eukaryotic cells. It was shown that in *P. aeruginosa*, the structural inner-rod protein PscI and the major component of the needle filament PscF activates the NLRC4 inflammasome (Figure 8), however the mechanism by which these two structural proteins of the injectisome enter the cell is still unknown (Grandjean et al., 2017). Inner rod proteins from many human pathogens (*S. enterica*, *Shigella flexneri* and *Burkholderia thailandensis*) were subsequently identified as potent NLRC4 activators (Reyes Ruiz et al., 2017), along with flagellin, the main component of the flagella responsible of bacterial motility, secreted by *S. enterica* in macrophages thanks to its SPI-1 T3SS (Franchi et al., 2006; Sun et al., 2007). On the other hand, the NLRP3 inflammasome is indirectly activated following membrane damages

induced by various pore-forming toxins (PFTs) like the aerolysin from *Aeromonas hydrophila* or the exolysin A (ExlA, described later) expressed by certain strains of *P. aeruginosa* (Figure 8) (Basso et al., 2017b; McCoy et al., 2010). Indeed, upon pore formation at the surface of the cell, the intracellular potassium (K^+) concentration decreases following K^+ efflux, triggering the activation of NLRP3 (Muñoz-Planillo et al., 2013).

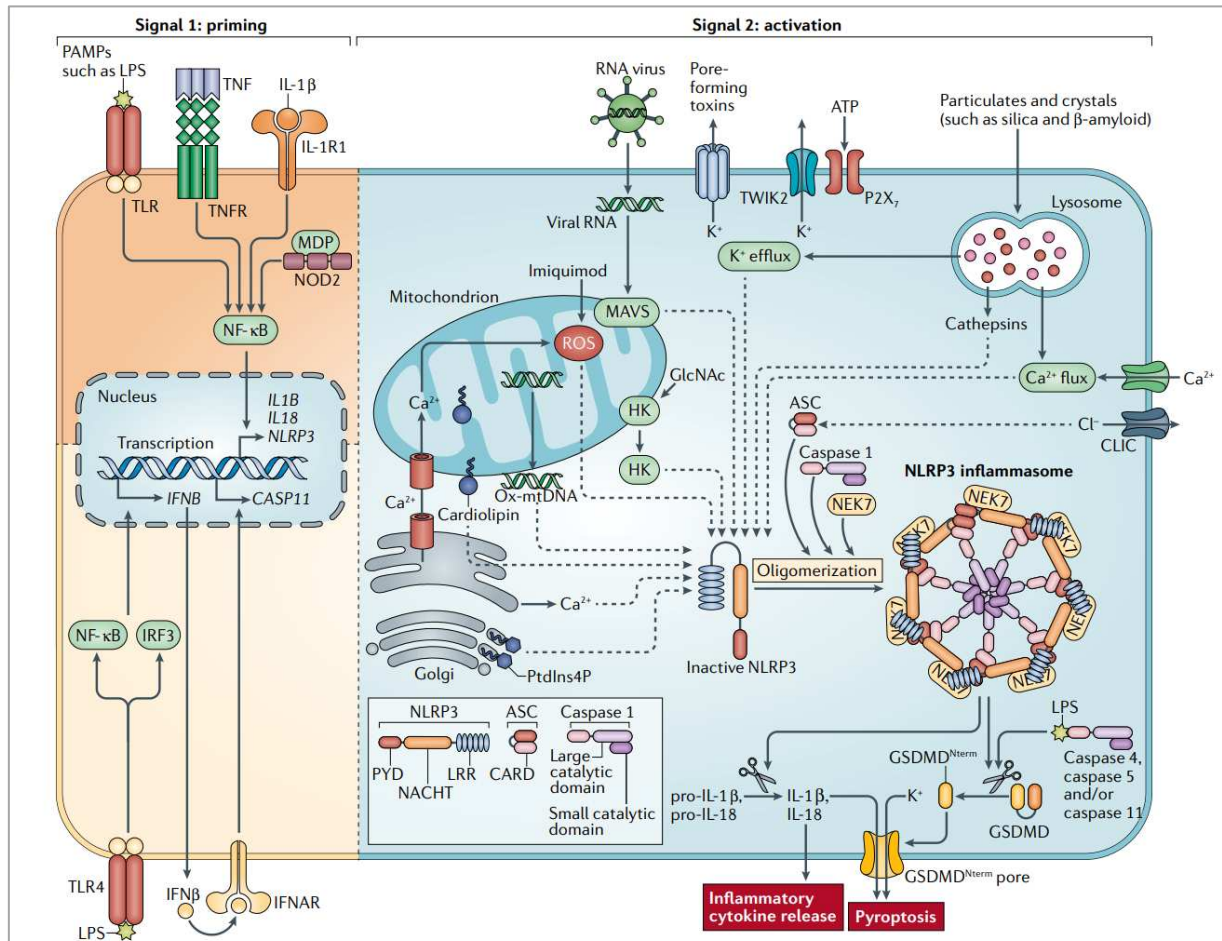


Figure 7: The complex and finely regulated process of inflammasome activation and pyroptosis. Following a primary signal through TLR activation, pro-inflammatory genes coding for pro-IL-1 β , pro-IL-18 and NLRP3 monomers are actively expressed. A second signal, specific for each type of NLRs, is then required to induce the formation of the inflammasome, responsible for the maturation of the two pro-inflammatory cytokines and their release in the extracellular space through pyroptosis. From (Swanson et al., 2019).

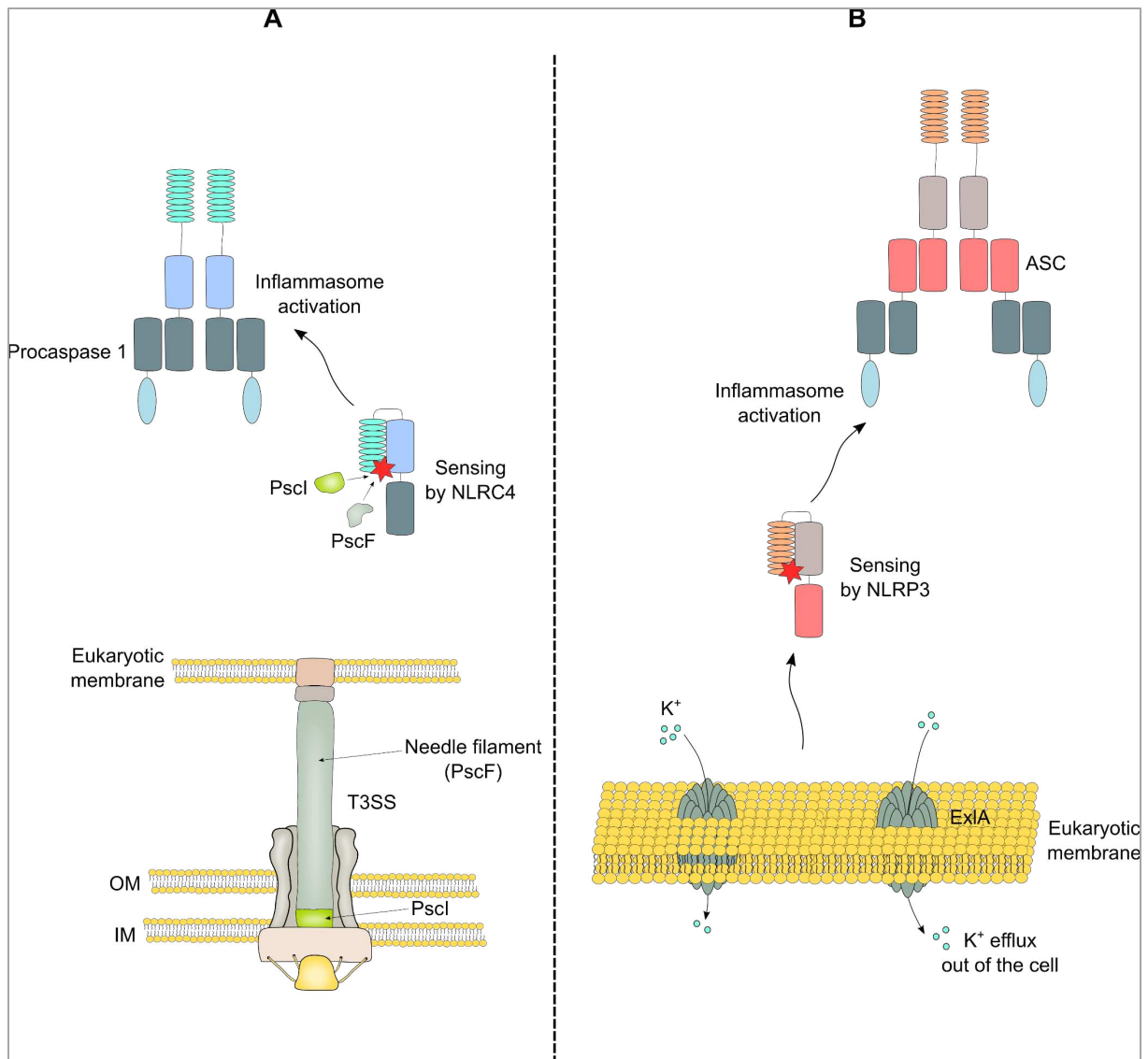


Figure 8: Inflammasome activation by *P. aeruginosa*. (A) T3SS effectors PscF or PscI are sensed by NLRC4, leading to inflammasome activation. (B) By forming a pore within the cell membrane, ExlA triggers the efflux of intracellular K⁺, an activating signal for NLRP3 inflammasome. In both (A) and (B), pyroptosis is activated, triggering cell death and release of pro-inflammatory mediators. OM: outer membrane. IM: Inner membrane.

Because inflammation is a milestone to establish an efficient immune response most of the time leading to bacterial clearance, pathogens have evolved ingenious mechanisms to evade immune surveillance. Many bacteria achieve this protective escape by blocking, at different levels, the intracellular signalization activated by TLRs dimerization. For instance, *Shigella flexneri* secrete the ubiquitin ligase IpaH9.8, targeting the essential signaling protein NEMO to proteasomal degradation, while OspG, secreted by the same pathogen, prevents

ubiquitinylation and subsequent degradation of the NF- κ B inhibitor I κ B α , maintaining the transcription factor in its inactive form (Reddick and Alto, 2014; Rosadini and Kagan, 2015). Also, the NleC metalloprotease found in attaching/effacing pathogens enteropathogenic *Escherichia coli* and *Citrobacter rodentium* inhibit NF- κ B by directly cleaving its p65 subunit (Hodgson et al., 2015). On their side, some *P. aeruginosa* strains are able to translocate the PumA protein within eukaryotic cells. The TIR domain of PumA binds to the key components MyD88 and TIRAP, which normally interacts with the cytosolic part of TLRs through their TIR motifs. By doing so, PumA inhibits NF- κ B activation and thus dampens the inflammatory response, promoting its virulence and survival in the host (Figure 9) (Imbert et al., 2017). Many cytoplasmic or nuclear effectors (e.g ATP, F-actin, Histones, DNA and RNA) are very potent alarmins sensed by various PRRs; necrotic material therefore triggers the activation of surrounding cells, which will initiate an inflammatory response. Thus, direct killing of sentinel cells using toxins is not an efficient way of immune evasion, unless an immunologically silent apoptosis-like cell death is induced. Indeed, upon apoptosis the cell membrane integrity is conserved, preventing the release of the cytoplasmic content.

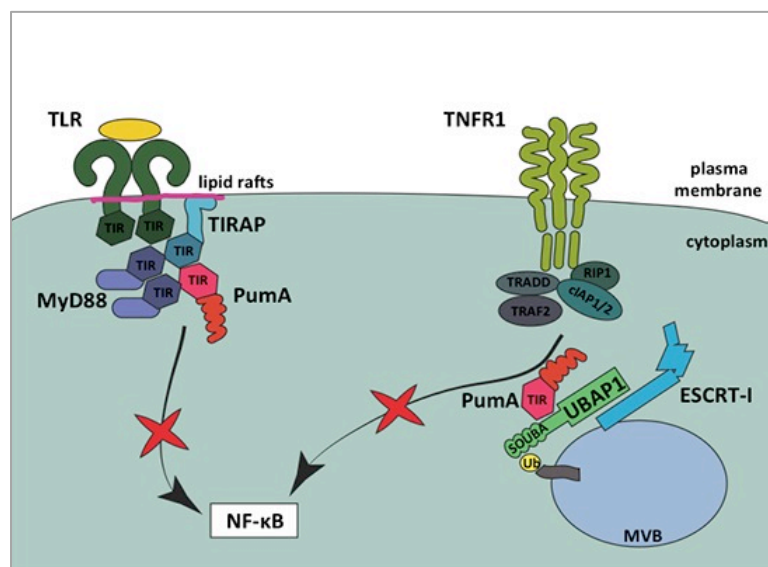


Figure 9: PumA from *P. aeruginosa* efficiently blocks NF- κ B activation. Thanks to its TIR domain, PumA directly binds to TIRAP and MyD88 through a TIR-TIR interaction, subsequently impairing their association with activated TLRs and downstream signaling required for NF- κ B activation. From (Imbert et al., 2017).

Inflammasome activation has been recognized to be essential for the protection against a plethora of important human pathogens (e.g *Bacillus anthracis*, *Escherichia coli*, *Francisella tularensis*, *Legionella pneumophila* or *Yersinia pestis*, among others) (Vladimer et al., 2013). However, bacteria have developed strategies to outsmart this immune detection. While components of the SPI-1 T3SS of *S. enterica* are sensed by NLRC4, the bacteria activates its second injectisome SPI-2 upon macrophage entry, which is not detected by the cell (Miao et al., 2010). *Yersinia* spp present a unique ability to inhibit inflammasome activation, by injecting YopK which interacts with and shields the tip of the T3SS needle (the translocon), preventing its detection by intracellular NLRs (Brodsky et al., 2010). It has also been demonstrated that for some bacterial species, activation of this sentinel system is deleterious for the host. For example, *P. aeruginosa* infections in knockout mice *Nlrc4(-/-)*, *caspase1/11(-/-)* *IL-1R(-/-)*, *IL-18(-/-)* or mice treated with caspase-1 inhibitors, are characterized by increased animal survival, reduced tissue damages and consistently lower bacterial load (Bouillot et al., 2020; Cohen and Prince, 2013; Faure et al., 2014; Schultz et al., 2003, 2002; Thakur et al., 2004; Véliz Rodriguez et al., 2012). Thus, pathogens can hijack the physiological role of NLRs to their benefit.

c) Resisting phagocytosis by resident macrophages and recruited neutrophils

In addition to the induction of the pro-inflammatory response, PRRs activation by a foreign invader triggers its phagocytosis by macrophages and recruited neutrophils. While TLRs signaling eventually enhances the phagocytic capacity of professional phagocytes, these membrane proteins are not considered as phagocytic receptors (Doyle et al., 2004). This function is mediated by other membrane-bound PRRs like Dectin-1, belonging to the C-type lectin receptors (CLRs) family, or various scavenger receptors. Pathogen's internalization is also efficiently promoted by opsonic phagocytic receptors like FcγRs and complement receptors (CRs), interacting with antibodies or complement proteins bound to the bacterial surface, respectively. Upon activation of phagocytic receptors, nucleation-promoting factors of the WASP family trigger the activation of the Arp2/3 complex essential for actin polymerization. Small GTPases Rac, Rho and Cdc42 are also recruited and activated, contributing to cytoskeleton and membrane remodeling. The cell membrane subsequently surrounds the bacterium, forming the phagocytic cup. Upon closure of this structure, the

pathogen is engulfed in the phagosome. This vacuole will then mature, to become highly bactericidal upon fusion with the lysosome (Figure 10). Indeed, within this newly formed phagolysosome, the pH is extremely acidic (between 5 to 5.5) thanks to the high number of V-ATPase present at its surface, translocating H⁺ inside this vacuole. This allows the activation of cathepsin D, a cysteine acidic protease with bactericidal activity. Reactive oxygen and nitrogen species (ROS and RNS) are massively produced in the phagolysosome because of the presence of NADPH oxidase, Myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS2). The pathogen is further harmed thanks to the action of various lipases, lactoferrin, defensins or the phospholipase sPLA2-IIA (Kinchen and Ravichandran, 2008; Rougerie et al., 2013; Thorne et al., 1976; Uribe-Querol and Rosales, 2017). As phagocytosis is one of the most potent way to eliminate foreign microorganisms, it is not surprising that human pathogens have developed smart mechanisms to cope with it.

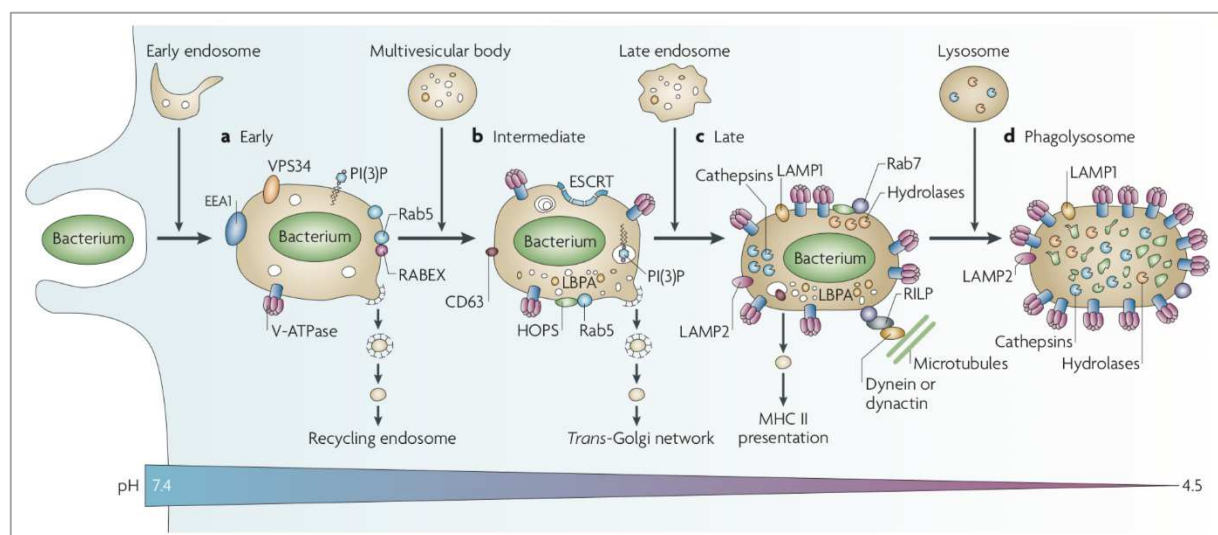


Figure 10: Phagosome formation and maturation. Following bacterial engulfment, the phagosome matures, becoming highly bactericidal upon fusion with lysosome, leading to the pathogen's lysis and degradation. From (Flannagan et al., 2009).

i) Reducing opsonization

As stated above, opsonins (mainly IgG, IgA and complement proteins) are potent enhancers of bacterial internalization through opsonophagocytosis. Foreign invaders can thus decrease the phagocytic potential of professional phagocytes by reducing their immunogenicity and limiting their interaction with opsonins. The capsule of *N. meningitidis* group B and *E. coli*

K1 is composed of polysialic acid, which are also present at the surface of eukaryotic cells, making them poorly immunogenic, thus limiting the production of specific antibodies targeting the bacterial surface (Stein et al., 2006). Thanks to its coagulase, *S. aureus* induces fibrin clotting at its surface, forming a shield around the bacteria composed of host material (McAdow et al., 2012). Bacteria also secrete molecular effectors able to degrade immunoglobulins, as illustrated by the elastase LasB or the protease IV of *P. aeruginosa* (Galdino et al., 2017). Conversion of the host's plasminogen into active plasmin by staphylokinase secreted by *S. aureus* promotes the degradation of surface-bound antibodies (Uribe-Querol and Rosales, 2017). Hijacking the physiological role of plasminogen in order to target opsonizing antibodies was also reported during *Francisella tularensis* infections (Crane et al., 2009). Protein A and G, present respectively at the surface of *S. aureus* and *Streptococcus pyogenes*, bind antibodies through their constant Fc regions, preventing their interaction with Fc receptors at the surface of phagocytic cells (Nezlin, 1998; Uribe-Querol and Rosales, 2017). Protein A secretion also induces immunoglobulin agglutination. Some proteins belonging to the complement system are also very potent opsonins, but the interaction between bacterial pathogens and this system will be discussed in more details later on.

ii) Killing phagocytes

A plethora of bacterial effectors/toxins are toxic to eukaryotic cells, including macrophages and neutrophils. *S. aureus* is very efficient at killing professional phagocytes by secreting different types of pore-forming leukocidins (e.g LukAB, LukED, PVL), which oligomerize at the host cell surface upon interaction with their specific receptors and induce its necrosis (do Vale et al., 2016). The pore-forming toxin streptolysin O secreted by group A *Streptococcus* also contributes to bacterial virulence, however by inducing cytochrome c release from the mitochondrial membrane, leading to macrophage and neutrophil apoptosis (Timmer et al., 2009). Gram-negative pathogens are also able to destroy professional phagocytes, mainly thanks to toxins secreted by their T3SS. Indeed, the two enteric bacteria *Shigella* spp and *Salmonella enterica* need a functional injectisome to induce neutrophil necrosis and macrophage killing by apoptosis or autophagy (Chen et al., 1996; François et al., 2000; Hernandez et al., 2003; Yang et al., 2015). Among the effectors secreted by *P.*

aeruginosa, the toxin ExoU, belonging to the phospholipase A₂ family, is the most toxic one and greatly contributes to bacterial pathogenicity *in vivo* (Finck-Barbançon et al., 1997; Howell et al., 2013; Shaver and Hauser, 2004). By degrading various types of lipids, ExoU induces membrane permeabilization and subsequent necrotic cell death (Sato et al., 2003). Upon infection, ExoU is mainly injected into phagocytic cells, impairing bacterial internalization and killing (Diaz et al., 2008; Diaz and Hauser, 2010). Representing around 30% of the clinical isolates (Sawa et al., 2014), ExoU-secreting strains are associated with increased infection severity (Schulert et al., 2003).

iii) Preventing actin polymerization

Another way used by pathogens to prevent their phagocytosis is to inhibit the formation of the phagocytic cup by blocking the actin polymerization machinery. In *P. aeruginosa*, this is mainly achieved by the T3SS-secreted exotoxin ExoS, a 49kDa protein firstly described in 1978 (Iglewski et al., 1978), expressed in approximately 60% of clinical isolates (Sawa et al., 2014). ExoS possesses two functional domains, one with GTPase Activating Protein (GAP) activity, and a C-terminal ADP-ribosyltransferase (ADPRT). Thanks to its GAP domain, ExoS activates the three small GTPases Rho, Rac and Cdc42, involved in actin polymerization when bound to GTP (Goehring et al., 1999; Pederson et al., 1999). By forcing these proteins to hydrolyze GTP, ExoS inhibits actin polymerization, leading to cell rounding and inhibition of internalization by professional phagocytic cells (Dacheux et al., 1999; Frithz-Lindsten et al., 1997). Thanks to its ADPRT domain, ExoS induces the transfer of ADP-ribose to various enzymes, inactivating them (Barbieri and Sun, 2004; Hauser, 2009). By targeting the GTPase Ras, involved in actin polymerization, this second domain of ExoS is also able to prevent *P. aeruginosa* phagocytosis *in vivo* (Rangel et al., 2014). ExoT and ExoY are two additional effectors secreted by *P. aeruginosa* to prevent actin cytoskeleton remodeling. While the former has a sequence 76% homologous to ExoS and has roughly the same function (except that its ADPRT efficiency is 1000 times lower and that it has a restricted number of specific substrates) (Barbieri and Sun, 2004; Hauser, 2009), the later binds F-actin and stabilizes actin microfilaments, preventing rearrangement of the cell cytoskeleton (Belyy et al., 2016). Targeting small GTPases is not specific to *P. aeruginosa* and has been described for other

human pathogens like *Yersinia* spp and *Burkholderia cenocepacia* (Figure 11) (Uribe-Querol and Rosales, 2017).

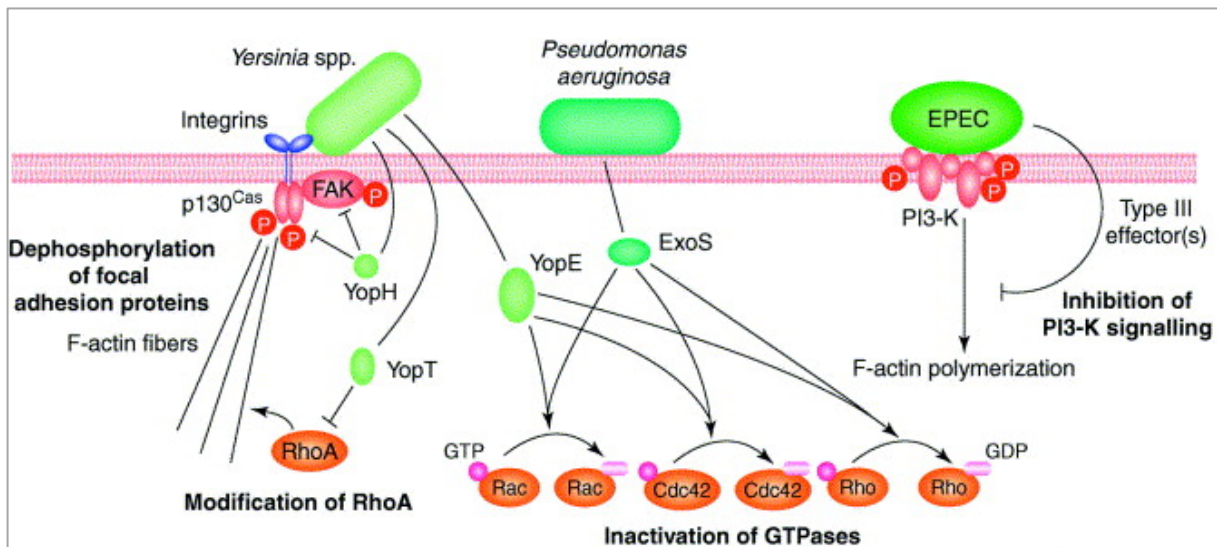


Figure 11: Inhibition of actin polymerization is a common strategy used by pathogens to prevent phagocytosis. Using specific effectors secreted thanks to their T3SS, Gram-negative bacteria interfere with essential actors controlling actin polymerization and subsequent membrane deformation, impeding the formation of the phagocytic cup. From (Celli and Finlay, 2002).

iv) Persisting within phagocytic cells

If the pathogen is not able to kill phagocytes or to prevent its internalization by immune cells, it will have to cope with the bactericidal environment of the phagolysosome. To do so, some bacteria prevent the fusion between lysosome and phagosome, like *N. meningitidis* which degrade lysosomal-associated proteins present at the surface of phagosome and essential for the fusion process. Harmful ROS can be neutralized by specialized enzymes like the super oxide dismutase, converting O_2^- into hydrogen peroxide H_2O_2 , which is subsequently used by the catalase to produce H_2O and O_2 . To resist lysis by phagolysosomal defensins, pathogens employ the same mechanisms stated earlier to cope with AMPs present in mucosae. Finally, the level of available iron within the phagolysosome is very low due to the presence of transferrin but also because of the active efflux of Fe^{2+} out of the vacuole mediated by the transporter NRAMP-1. Due to the essential role of this element for bacterial survival, pathogens secrete siderophores with very high affinity for Fe^{2+} able to scavenge iron bound to the host proteins like transferrin. For instance, *P. aeruginosa* releases the two potent siderophores

pyochelin and pyoverdine, potentially preventing iron depletion in this harmful environment, however their involvement in survival within phagolysosome has not been studied. All these mechanisms of resistance, which are not exhaustively presented here, were very well reviewed by (Uribe-Querol and Rosales, 2017).

d) Breaking through the epithelial and endothelial mechanical barriers

Mechanical barriers also play a major role in limiting pathogen entry or dissemination within the host. This function is achieved by the epithelium, separating the inside of the organism with the external environment, and the endothelium lying between the circulating blood and tissues. In some cases, the epithelium is the first line of defense encountered by the pathogen, before the innate immune effectors (e.g skin epidermidis), but this is not always the case. For instance, in the lungs, alveolar macrophages are present in the external environment and face invaders before they cross the pneumocytes' layer and enter within tissues.

The structure of epithelia varies depending of their location within the body. They are formed by polarized cells, presenting an apical pole directed toward the external environment, and a basolateral part interacting with neighboring cells and the basement membrane on which they adhere. Along with their different architectures, epithelia do not have the same function. Indeed, while the role of the stratified squamous epithelium forming the skin epidermidis is to protect the body from mechanical abrasion, the simple columnar intestinal epithelium allows the passage of water and essential nutrients from the gut while preventing the entry of harmful substances. At mucosal surfaces, goblet cells specialized in mucus production release mucins in the external environment, coating the epithelium with an extra mechanical barrier. The mucus also contains AMPs secreted by epithelial cells. The mechanical protection conferred by the epithelium against pathogen entry relies on the intercellular junctions allowing epithelial cells to be in tight contact, preventing passage of particles between them. Four main types of junctions maintain epithelial cells together (Figure 12).

Tight junctions are located near the apical pole of the cells. They allow the selective passage of small molecules needed by the organism like water, ions and various macromolecules. They are formed mainly thanks to homophilic interactions between claudins.

These junctions also contain occludin and junctional adhesion molecules (JAMs), while the zona occludens proteins bridge actin cytoskeleton and these membrane-bound effectors (Campbell et al., 2017; Shin and Margolis, 2006).

Adherens junctions are found below the tight junctions and are formed by clustering and association of epithelial cadherin (E-cadherin). Like for the proteins forming the tight junctions, E-cadherin interact with the actin cytoskeleton through adaptor proteins, which are in this case α - and β -catenin. Nectins proteins are also found at adherens junctions, even though the connection they create between cells is not as strong as the one involving E-cadherin (Campbell et al., 2017).

Another type of structure by which epithelial cells are linked together is the desmosome. These disc-like structures are mainly composed of the desmosomal cadherins desmogleins and desmocollins, interacting with homologous effectors on neighboring cells. These proteins are tightly bound to intermediate filaments on their cytosolic part. Interaction between adhesive molecules and the cytoskeleton is a common feature of intercellular junctions, allowing the cells to resist mechanical stress (Kowalczyk and Green, 2013).

Finally, gap junctions, found near the basal pole of the cell, are formed by the oligomerization of connexins, creating a connexon at the cell membrane. Upon interaction between two connexons from adjacent cells, an intercellular channel is formed, connecting the two cytoplasm. These canals allow the transfer of small molecules and ions from one cell to another (Goodenough and Paul, 2009).

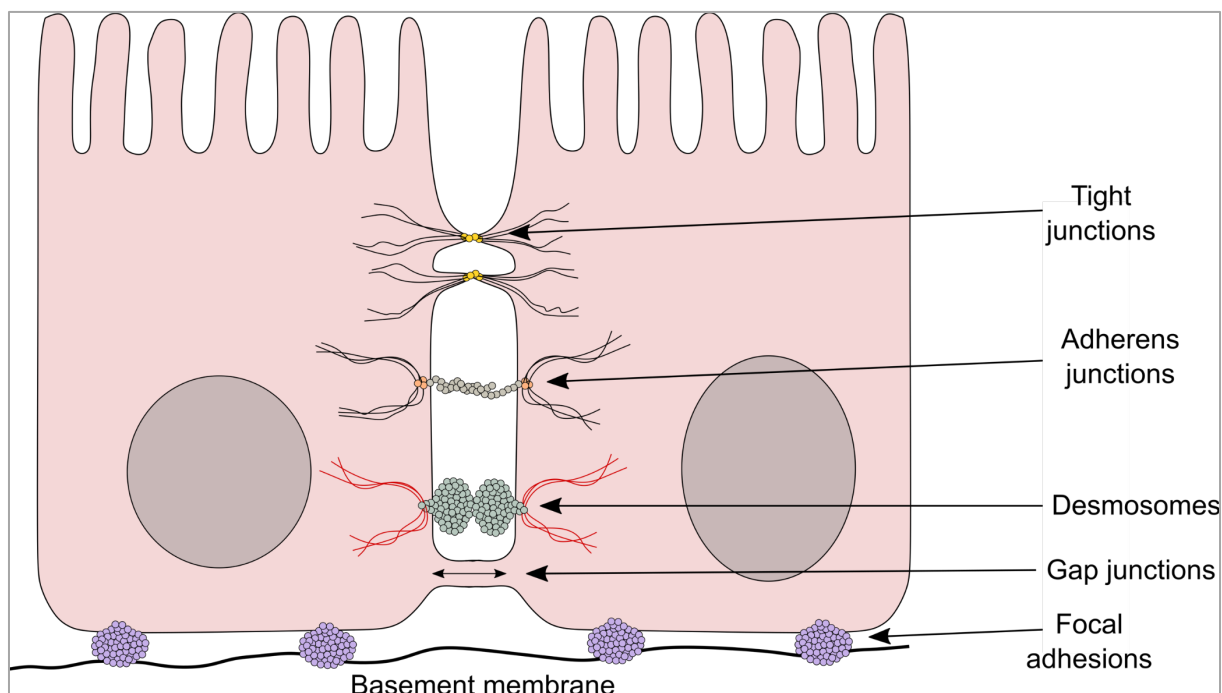


Figure 12: The different types of intercellular junctions connecting epithelial cells and enabling them to form an impermeable barrier against bacteria.

The endothelium is formed by a monolayer of endothelial cells maintaining the blood in a closed circuit, while allowing the selective passage of essential nutrients and gas from the blood to the surrounding tissues. Like the epithelium, the endothelium properties vary throughout the entire vascular system (arteries, veins, capillaries and postcapillary venules). As an example, its permeability in the brain is greatly reduced as the blood brain-barrier forms a very selective and protective separation between the circulation and the central nervous system. On the opposite, the endothelium forming postcapillary venules is much more permissive for various transfers between blood and tissues (Aird, 2012; Stamatovic et al., 2008). Like within the epithelium, endothelial cells are connected together thanks to intercellular junctions. Most of them are closely related to the one described with epithelial cells, however no desmosomes are found in the endothelium. A few adhesive proteins are specific to endothelial cells, which are the VE-cadherin and the claudin-5, involved in adherens and tight junctions, respectively (Figure 13) (Wallez and Huber, 2008).

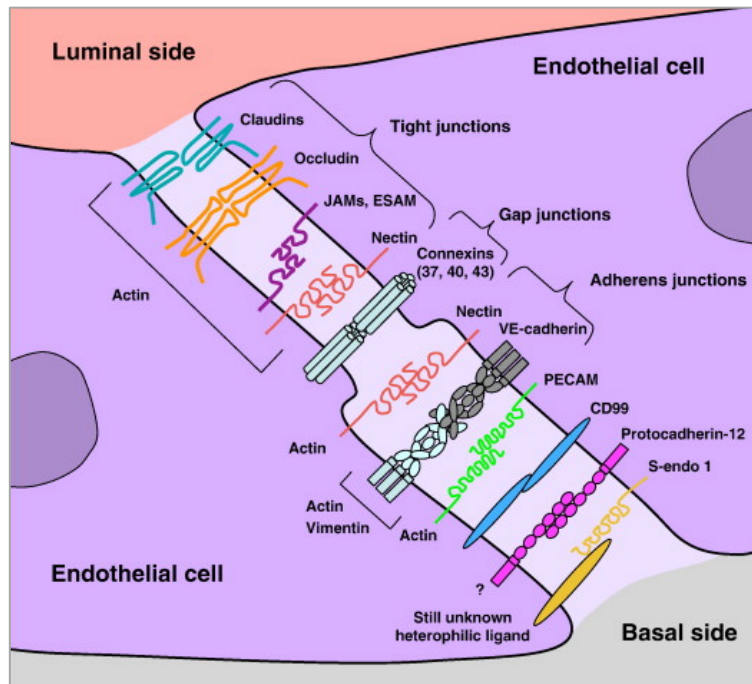


Figure 13: Intercellular junctions connecting endothelial cells together. From (Wallez and Huber, 2008).

In order to reach the bloodstream and disseminate within the organism, pathogens have to cross these two cell barriers, representing an important challenge. Bacteria achieve that process through three main mechanisms.

i) Cell killing

Firstly, pathogens can kill epithelial or endothelial cells thanks to their toxic virulence factors. Using its T3SS, the opportunistic pathogen *P. aeruginosa* is unable to induce cell death by injecting its exotoxins at the apical pole of epithelial cells. However, upon cell division or senescence, cell to cell junctions are transiently opened, allowing bacteria to deliver their toxic effectors at the baso-lateral side of the cell membrane, efficiently inducing its death and causing the barrier to open, allowing the pathogen to transmigrate (Golovkine et al., 2017, 2016). Inhibition of the small GTPases Rho, Rac and Cdc42 in endothelial cells following injections of the two T3SS effectors ExoS and ExoT also induces cell retraction, causing the endothelium to break (Huber et al., 2014). Other toxins like pneumolysin and β -hemolysin respectively

secreted by *S. pneumonia* and *Streptococcus agalactiae*, induce apoptosis of endothelial cells, thus disrupting the blood-brain barrier (Doran et al., 2013).

ii) Targeting cell to cell junctions

The second mechanism by which pathogens break through epithelia and endothelia relies on the disruption of intercellular junctions. For instance, once in the gut, *Clostridium perfringens* secretes the enterotoxin CPE, able to enter the cell *via* a pore-forming mechanism, enabling it to interact with high affinity with claudin-3 and 4 essential for junctions maintenance. Upon binding of CPE to its targets, the two claudins are proteolyzed, increasing the intestinal epithelium permeability (Doran et al., 2013; Sonoda et al., 1999). Bacterial proteases can also directly target intercellular junctions, as observed for the elastase LasB secreted by *P. aeruginosa*. LasB specifically cleaves VE-cadherin of endothelial adherens junctions, increasing the dissemination of the pathogen *in vivo* (Golovkine et al., 2014). In addition to the utilization of its own protease, *P. aeruginosa* is also able to induce E and VE-cadherin cleavage by activating the host protease ADAM10. Indeed, insertion of the pore-forming toxin ExlA within epithelial and endothelial cells' membranes induces a massive entry of Ca²⁺ within cells. This increase in calcium concentration activates the protease ADAM10 to cleave cadherins, likely contributing to bacterial dissemination within the organism (Figure 14) (Bouillot et al., 2017; Reboud et al., 2017).

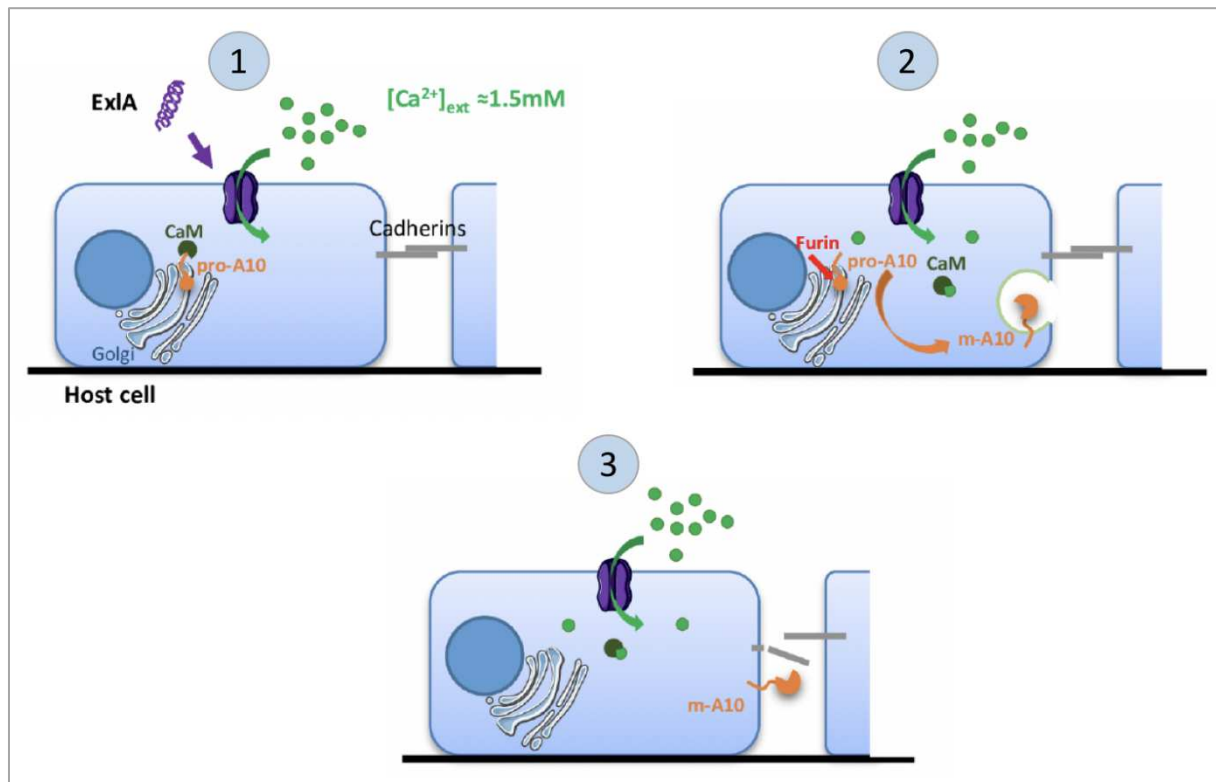


Figure 14: *P. aeruginosa* ExlA induces cadherin cleavage by the host protease ADAM10. By forming a pore within the cell membrane, ExlA allows the extracellular Ca^{2+} to enter the cells. Ca^{2+} subsequently binds calmodulin, initially associated to inactive protease pro-ADAM10. The protease is subsequently targeted to the membrane in its active form. Once at the cell surface, it cleaves cadherins that are part of adherens junctions, weakening the epithelial and endothelial barriers and favoring bacterial transmigration. Adapted from (Reboud et al., 2017).

iii) Use of Trojan horses

Finally, engulfed bacteria able to resist the harsh environment of phagolysosome can take advantages of the migratory potential of professional phagocytes to pass through the different cellular barriers. The Gram-negative pathogen *Salmonella* spp. delivers, through its T3SS, the effector SrfH within macrophages recruited to the lumen of the intestine. By interacting with the protein TRIP6 controlling cytoskeletal rearrangement and cell motility, SrfH promotes migration of infected macrophages through the gut epithelium and the endothelial barrier to reach the bloodstream leading to septicemia (Lin and Lin, 2011; Worley et al., 2006). This so called ‘trojan horse’ mechanism of dissemination has also been reported for the Gram-positive pathogen *Listeria monocytogenes*, however the molecular mechanism in place is still poorly understood (Drevets, 1999; Santiago-Tirado and Doering, 2017).

Chapter II

The innate immune system in the blood

The blood is normally sterile firstly because it is not exposed to the external environment and is protected by the different defenses described above, but also because it is highly microbicidal. Indeed, while its rich nutritious composition could theoretically support bacterial growth, the presence of cellular and humoral innate immune effectors renders the blood mostly non-permissive to microbial development.

1) Leukocytes of the blood and their role against bacterial pathogens

Different immune cell types are found in the circulation, as presented in [Table 1](#) along with their respective frequencies in healthy adults and their principal functions.

Cell types and numeration	Subpopulations	Frequencies (% of total leukocytes)	Main functions
Granulocytes ($3.1-5.3 \times 10^6/\text{mL}$)	Neutrophils	40-65	Eliminate bacterial and viral pathogens, recruited at the site of infections
	Eosinophils	0.1-2	Fight parasites using their eosinophilic granules
	Basophils	0.1-2	Induce inflammation by histamine release
Lymphocytes ($1.5-3.9 \times 10^6/\text{mL}$)	B cells	3-10	Release specific antibodies
	T cells (CD4 ⁺)	10-20	Initiate and assist the adaptive immune response
	T cells (CD8 ⁺)	3-10	Destroys cells infected by intracellular pathogens
	Natural Killer cells	2-5	
$\gamma\delta$ T cells	1-5	Link the innate and the adaptive immune responses	
Monocytes ($2.3-7.8 \times 10^5/\text{mL}$)	-	3-10	Eliminate bacterial and viral pathogens, recruited at the site of infections where they differentiate into fully competent macrophages
Dendritic cells ($3.9-7.8 \times 10^4/\text{mL}$)	-	0.5-1	Reach the tissues to sense the presence of pathogens, activate the adaptive immune response

Table 1: The different subpopulations of leukocytes in human whole blood, their frequencies and their main functions. Note that the values are approximations as important variations can be observed between two healthy individuals.

This work focuses on an acute context of infection, the function of leukocytes mostly involved in adaptive immunity will therefore not be examined. It is however important to note that in the case of chronic infection, like in cystic fibrosis (CF) patients infected by *P. aeruginosa*, a strong adaptive humoral response is triggered by the pathogen. This reaction is characterized by high titers of specific antibodies directed against various bacterial virulence factors like elastase, LPS and flagella, and formation of immune complexes deleterious for the patient and the lung functions. These complexes accumulate in the lungs, inducing complement and neutrophil activation and subsequent damages to surrounding tissues, decreasing lung functions and contributing to the persistence of the infection (Jensen et al., 2010; Mauch et al., 2018).

Neutrophils and monocytes are the main white blood cells of the circulation directly engaged in the fight against bacterial pathogens, the function of these two cell types will thus be further discussed in this section. These two myeloid cells originate from the bone marrow, following differentiation of the granulocyte-macrophage progenitor in monocyte or myeloblast, the later requiring further signals to generate neutrophils (Figure 15). Differentiations of progenitor cells into one lineage or another is regulated by cytokines belonging to the colony-stimulating factors (Metcalf, 2008). Once generated in the bone marrow, these cells join the circulation where they start patrolling, seeking for activating signals like chemokines and proinflammatory cytokines or direct interaction with bloodborne pathogens. While neutrophils do not exit the bloodstream in steady state, monocytes actively join the tissues in which they differentiate into macrophages or DCs, to replenish certain populations of resident phagocytic cells (Jakubzick et al., 2017).

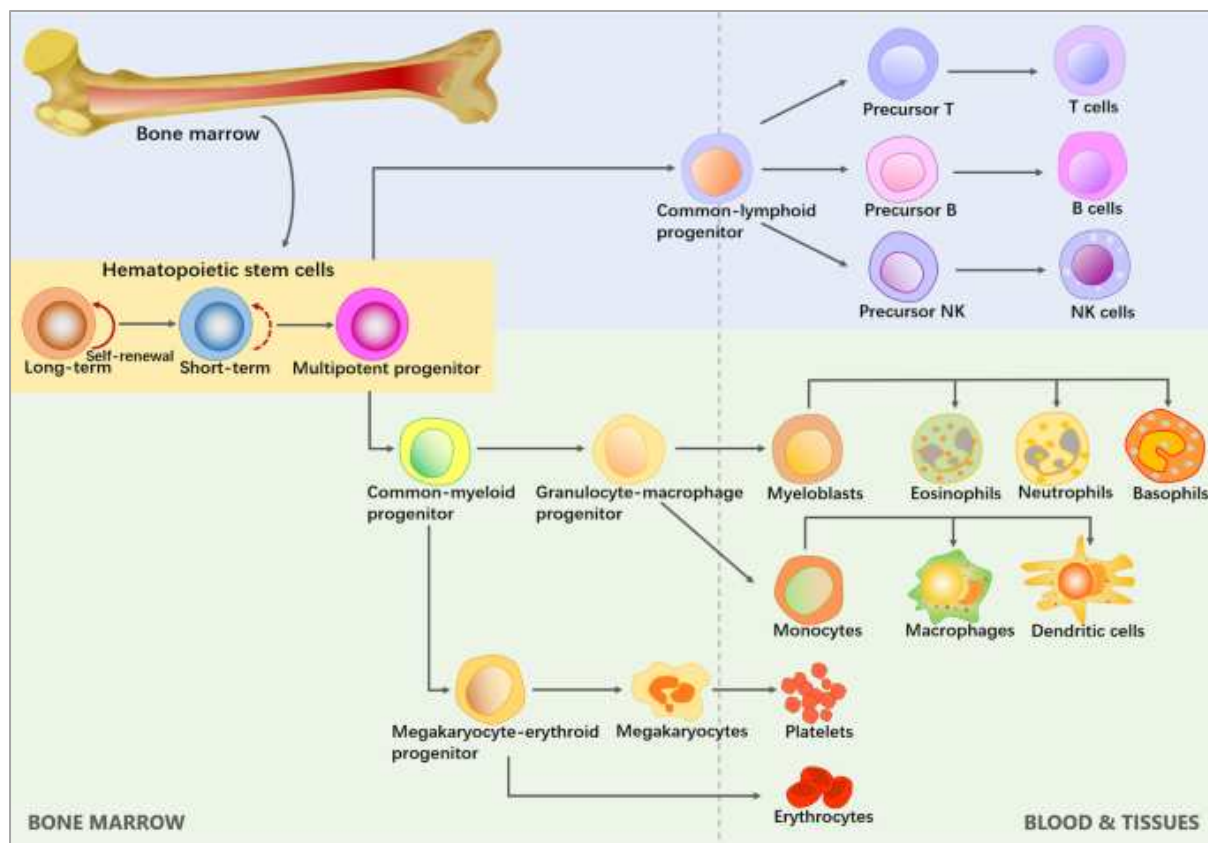


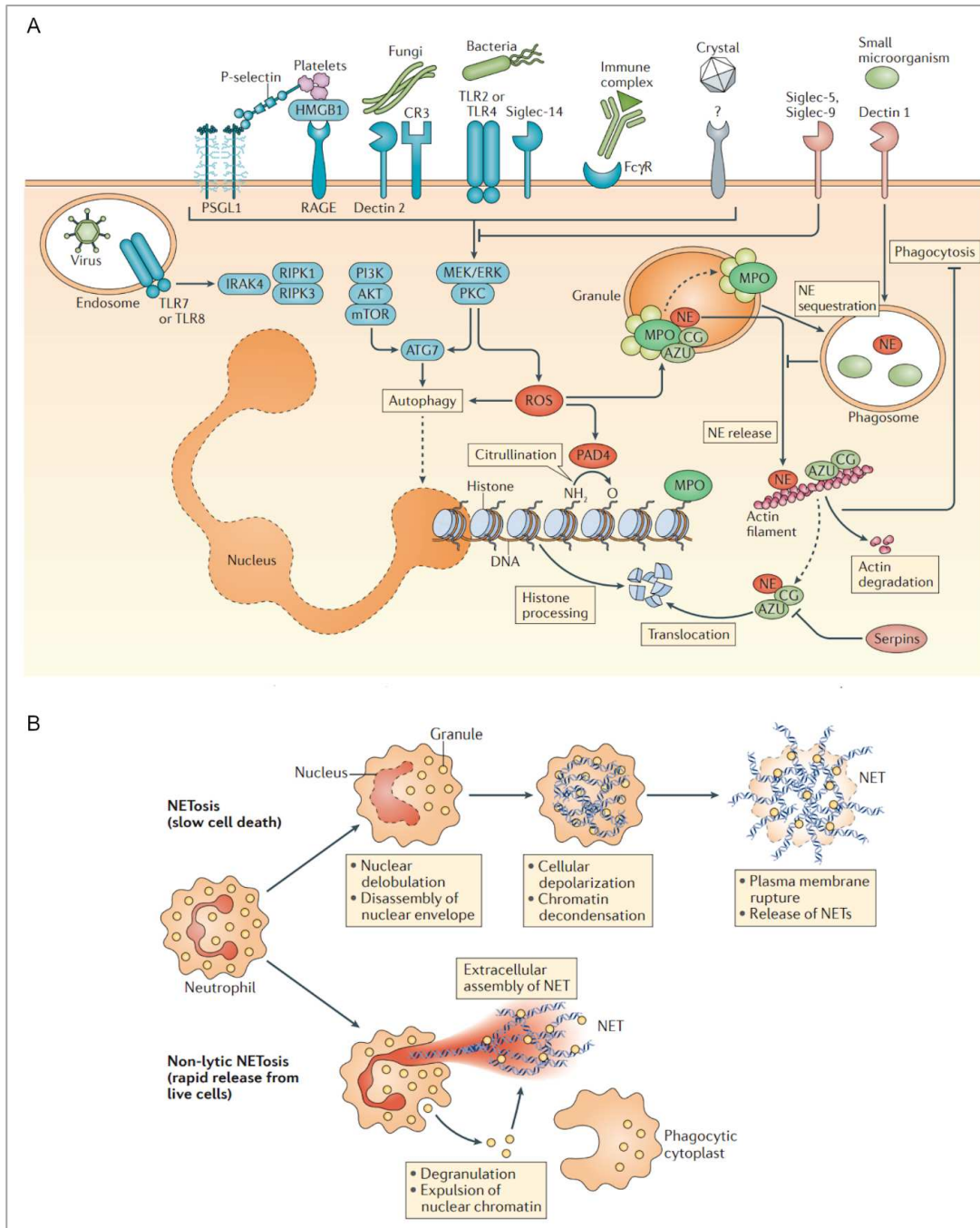
Figure 15: The different hematopoietic progenitors controlling the production of all the cellular effectors found in the blood. From (Zhang et al., 2019).

Once in contact with a foreign invader, either in the circulation or within infected tissues following their recruitment, neutrophils and monocytes are supposed to phagocytose the pathogen, as previously described. The essentiality of these cells upon infection was confirmed following their recruitment at pathogens' entry site (Koh et al., 2009; Serbina et al., 2008), however their bactericidal potential within the blood circulation has been less studied and might differ from what was observed in tissues depending of the bacterial species. For instance, neutrophils in the lung microvasculature are able to rapidly engulf *S. aureus*, *E. coli* or *S. pneumoniae*, but are poorly able to internalize *P. aeruginosa* circulating in the blood, while these phagocytes are absolutely required to fight this same pathogen within lungs (Koh et al., 2009; Thanabalasuriar et al., 2017). In addition to phagocytosis, these two cells are able to release extracellular traps (ETs), which are web-like structures composed of DNA coated with various antibacterial effectors. This phenomenon was firstly described in 2004 by (Brinkmann et al., 2004), who identified that neutrophil extracellular traps (NETs) were able to trap and kill pathogenic bacteria. Since that discovery, many studies aimed at better understanding this

elegant mechanism of bacterial clearance. It is now known that various ligands (e.g immune complexes or the siderophore pyocyanin secreted by *P. aeruginosa*) stimulate neutrophils by inducing a complex signalization following binding to their cognate receptors (e.g CLR, TLR, FcγR), subsequently activating the MEK/ERK kinases and leading to ROS production in the cell (Papayannopoulos, 2018; Rada et al., 2013). Subsequent downstream events can eventually lead to chromatin decondensation and association with diverse components present in the cell cytoplasm and granules (Figure 16A). More than 20 different proteins have been described to associate with DNA in this context, among them the MPO, the two proteases neutrophil elastase (NE) and cathepsin G, defensins and cathelicidins, all with potent antimicrobial activity. Finally, this DNA/protein mesh is released in the extracellular space upon cytoplasmic membrane disruption, by a type of cell death known as NETosis. A growing body of evidences tends to show that vital NETosis also occurs *in vivo*, during which the nuclear content of the cell is rapidly expelled in the surrounding environment, leaving an anucleated neutrophil (called cytoplast) still able to phagocytose and kill bacteria (Figure 16B) (Papayannopoulos, 2018; Pilszczek et al., 2010; Yipp et al., 2012; Yipp and Kubes, 2013). In addition to the different effectors decorating these structures able to trap bacteria, DNA *per se* is bactericidal through cation chelation and subsequent damages induced to the bacterial membrane (Halverson et al., 2015). It is important to note that the nature of the signal detected by the neutrophil dictates whether the pathogen will be phagocytosed or if NETs will be released, as these two mechanisms antagonize each other (Figure 16A). Other cell types including monocytes and macrophages were also shown to produce extracellular traps analogous to NETs, with similar functions against pathogens (Aulik et al., 2012; Doster et al., 2018; Granger et al., 2017). *In vivo*, even though NETs seem to be efficient at trapping bacteria in the blood (Clark et al., 2007), their contribution in the fight against pathogens seems selective regarding the bacterial species. For instance, preventing NETs in mice (either by using DNase treatment or PAD4^{-/-} animals lacking the enzyme leading to chromatin decondensation) had no effect on the outcome of *Burkholderia pseudomallei* or polymicrobial bacteremia (de Jong et al., 2014; Martinod et al., 2015). By contrast, the same prevention of NETs release (PAD4^{-/-} mice) increased the susceptibility of animals infected with *S. pyogenes* and impaired *S. flexneri* killing by PAD4^{-/-} neutrophils (Li et al., 2010). This could likely be due to intrinsic differences between pathogens to cope with these deadly structures. Indeed, bacteria have developed ways to prevent ETs-mediated killing. Firstly, pathogens can prevent the activation of ETs release by interfering with

the signaling cascade governing this process. By presenting sialic acids at their surface, either produced by the bacteria or acquired from the host, group A streptococci and *P. aeruginosa* engage the receptor Siglec-9, inhibiting ROS production essential for NETs release (Figure 16) (Khatua et al., 2012; Secundino et al., 2016), explaining why the later pathogen is a weak inducer of NETs (Halverson et al., 2015). In case this first step of signaling interference does not prevent DNA release, certain bacteria are able to degrade these web-like structures thanks to the secretion of nucleases. This was well described for the two Gram-positive pathogens *S. pyogenes* and *S. aureus*, respectively synthesizing the effectors Sda1 and NucA (Buchanan et al., 2006; Storisteanu et al., 2017). Finally, production of a positively charged polysaccharidic capsule around the bacterial cell reduces its interaction with cationic effectors bound to DNA fibers, thus preventing trapping of the *S. pneumoniae* within these filaments (Wartha et al., 2007).

Due to the toxic potential of the molecules coating NETs, it is not surprising that NETosis was shown to cause tissue injury in certain settings. Indeed, in a model of bacteremia, endothelial cell death was induced following NETs release due to the interaction between neutrophils and activated platelets (Clark et al., 2007), while preventing the formation of these structures greatly reduced tissue injury in the liver (Kolaczowska et al., 2015). Because the normally intracellular cytoplasmic content becomes exposed during NETosis, the risk of self-antigen recognition by the immune system can't be overlooked. In this line, evidences have emerged linking NETs with autoimmune and other noninfectious diseases (Jorch and Kuberski, 2017).



The last mechanism specific to granulocytes allowing them to destroy bacteria is the process of degranulation. Four types of secretory granules are found within neutrophils. These structures are full of bactericidal components like elastase, MPO, cathepsins or defensins, with azurophilic (also known as primary) granules containing most of these effectors. Upon neutrophil stimulation, secretory granules are targeted to the cytoplasmic membrane, a process dependent on actin polymerization. After fusion with the cytoplasmic membrane, their toxic content is released in the vicinity of the cell, targeting neighboring pathogens (Lacy, 2006). In addition to mechanisms previously described to resist cationic defensins, pathogens can release inhibitors targeting neutrophil proteases. Among them, the periplasmic ecotin of *E. coli*, *Y. pestis* and *P. aeruginosa* efficiently inhibits the activity of the two major neutrophil serine proteases elastase and cathepsin G (Eggers et al., 2004; Stapels et al., 2015). Protease inhibitors homologous to human α -macroglobulin have also been identified in some Gram-negative bacteria, like MagD synthesized by *P. aeruginosa*, however their importance during infection and interaction with neutrophils has not been proved yet (Robert-Genthon et al., 2013; Stapels et al., 2015). In Gram-positive pathogens, the three extracellular adherence protein Eap, EapH1 and EapH2 from *S. aureus* specifically inhibit elastase and cathepsin G, favoring bacterial virulence in mice (Stapels et al., 2014).

2) The complement system

Within the circulation, a cocktail of various molecules renders the blood non-permissive to bacterial growth. These protective effectors comprise circulating AMPs, the two iron-binding proteins transferrin and lactoferrin which sequester free iron, and the major immune component: the complement system (Cassat and Skaar, 2013; Jendeberg et al., 2013). Due to the fact that complement represents the main arm of the humoral innate immunity in the particular environment of the blood, the functioning of this system will be described, along with the complex interplay between these proteins and bacterial pathogens, especially *P. aeruginosa*.

a) General description of the system

The complement system is an ancient primitive immune defense found in vertebrates and some invertebrate species, which gained in complexity along with the evolution since the appearance of cartilaginous fish (Sunyer and Lambris, 1998; Zhu et al., 2005). It comprises more than 30 different proteins, involved in the response against invading microorganisms, as well as in physiological processes occurring in steady state like removal of apoptotic cells or tissue regeneration (Clark et al., 2008; Merle et al., 2015; Mevorach et al., 1998). These proteins are mainly found in the blood but also within tissues, even though the activity of this system seems to be maximal in the circulation (Bolger et al., 2007). The complement cascade, a limited proteolysis event leading to zymogens' activation, can be triggered by three different pathways depending on the activating signals (Figure 17). The activation of the classical pathway (CP) is mediated through the interaction between the complex C1q-C1s-C1r and antibodies or pentraxins bound to their target (e.g pathogens), but also when C1q interacts directly with Pathogen Associated Molecular Patterns like the LPS of Gram-negative bacteria (Roumenina et al., 2008). The lectin pathway (LP), is activated following the binding of the Mannose-Binding Lectin (MBL) to mannose residues on sugars expressed by foreign microorganisms and subsequent activation of the two serine proteases MASP-1 and MASP-2 (Merle et al., 2015). Finally, the alternative pathway (AP) is constitutively active at a low level, through a tick-over mechanism, which is amplified upon pathogen detection (Merle et al., 2015). Following complement activation, regardless of the pathway, the central molecules C3b and C5b are produced, after cleavage of C3 and C5 by the C3 and C5 convertases, respectively. When bound to hydroxyl groups at the surface of a pathogen, C3b contributes to the generation of C5b as it is a component of the C5 convertase. Also, upon specific cleavage of C3b by regulatory proteins from the host, the inactivated fragment iC3b is generated at the bacterial membrane, which is recognized by the specific complement receptors CR3 and CR4 expressed by professional phagocytic cells, greatly enhancing their engulfment capacities, a process known as opsonophagocytosis. Before being cleaved, C3b mediates opsonophagocytosis through its interaction with CR1, expressed at the surface of neutrophils and macrophages. If the pathogen does not interfere with the complement cascade, the membrane attack complex (MAC) is formed. This complex, composed of C5b-C9, is assembled at the pathogen's surface and inserts within its membrane, leading to the formation of pores subsequently killing

susceptible invaders by lysis. Upon C3 and C5 cleavage, anaphylatoxins C3a and C5a are released, promoting the inflammatory response, along with the recruitment and the activation of immune cells (e.g neutrophils) at the site of infection (Klos et al., 2009). C5a is also able to induce NETs release (de Bont et al., 2019; Martinelli et al., 2004). Thanks to its involvement in diverse mechanisms of pathogen elimination, this versatile system represents an efficient barrier against many invaders. However, due to its potential harmful effects on the host if not tightly balanced, many different complement regulators are found either in the fluid phase or bound at the host cells' membrane, which are valuable targets used by pathogens to protect themselves against complement-mediated lysis or opsonophagocytosis.

Negative complement regulators are found both in the fluid phase or bound at the surface of the host's cells. Their function is to prevent undesirable and deleterious activation of the system at the cell surface. C4 binding protein (C4BP), decay acceleration factor (DAF) and complement receptor 1 (CR1) are membrane-bound regulators able to destabilize the C3 and C5 convertases (C4BP is also found in the fluid phase). DAF and CR1 are also cofactors of the complement factor I (FI), a serine protease circulating in the blood which cleaves C3b and C4b when it interact with its cofactors (Noris and Remuzzi, 2013). C3b cleavage generates iC3b, promoting opsonophagocytosis (see above) but unable to interact with C4bC2a to form the classical C5 convertase. Factor H (FH) and factor H-like protein-1 (FHL-1), found in the fluid phase as well, are also cofactors for the proteolytic action of FI on C3b, but are in addition able to bind C3b to prevent its interaction with factor B, an association essential for the formation of the alternative C3 convertase (Figure 17). Downstream of the amplification loops controlled by the C3 convertase, which produces more and more C3b, oligomerization of C9 and subsequent formation of the MAC at the membrane is a major threat for eukaryotic cells. Specific effectors are thus able to disassemble potentially harmful MAC. Vitronectin, clusterin (both found in the fluid phase) and the membrane-bound protein CD59 (also known as

protectin), impede the insertion of the C5b-C8 complex within the membrane, preventing the recruitment and oligomerization of C9 (Merle et al., 2015).

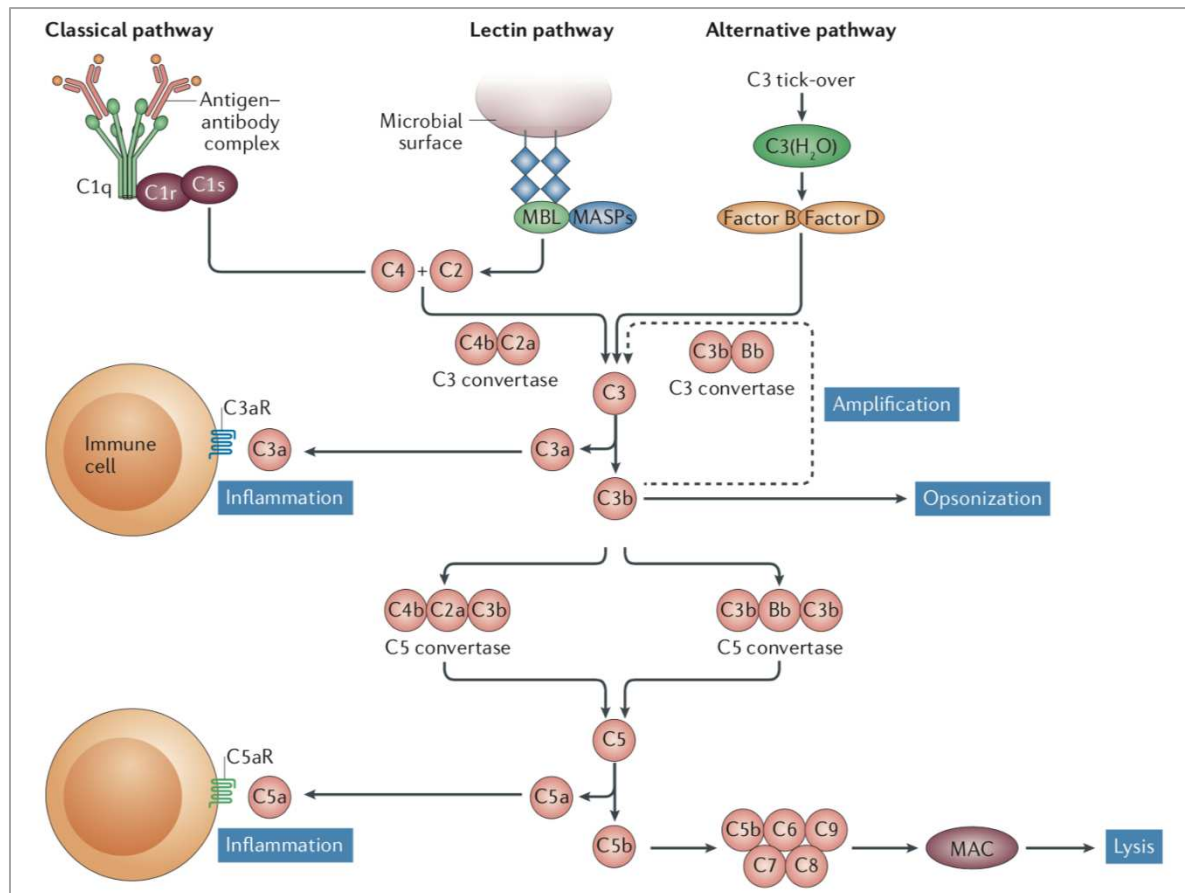


Figure 17: The complement system, its activation routes and the function of its effectors. From (Trouw et al., 2017).

b) Bacterial strategies to escape complement-mediated killing

Withstanding the deadly action of the complement is a prerequisite for bacteria to navigate within the circulation and disseminate within the organism. Even though this system is complex and activated by many different signals, making it potentially hard to be inhibited, microorganisms have evolved mechanisms to cope with its bactericidal potential. Similar mechanisms are used by both Gram-negative and Gram-positive pathogens, however their finality might differ. Indeed, due to their thick layer of PG composing their cell wall, Gram-positive bacteria are naturally resistant to the lytic action of the complement (Joiner et al., 1983). For these microorganisms, interfering with the complement cascade will thus confer a

protection toward opsonophagocytosis, rather than direct lysis. On the other hand, Gram-negative pathogens are susceptible to MAC insertion within their membrane, even though the exact mechanism of bacterial death induced by this event is not completely understood. Because the Gram-negative envelope is thicker than the size of the MAC, speculative models have been proposed to explain the complement-mediated disruption of both the inner and outer membranes. In the first model, the MAC would insert at Bayer's junctions, which are membrane regions in which the inner and outer membranes are in close contact (Berends et al., 2014). The second model proposes that MAC inserted at the OM allows the passage of lysozyme and complement proteins in the periplasmic space, the former degrading the thin layer of PG giving access to the IM for the complement proteins which subsequently assemble to form a deadly MAC at the IM (Berends et al., 2014). It was also hypothesized that following passage through the first MAC at the OM, complement proteins could readily pass through PG pores to reach the IM, without need for lysozyme-mediated PG degradation (Berends et al., 2014).

The strategy which is the most used by bacteria to prevent complement-mediated killing is the recruitment of negative regulators at the pathogen's surface (Kraiczy and Würzner, 2006; Lambris et al., 2008). These inhibitors of the complement cascade are found in high quantities and share common domains, meaning that bacteria can easily recruit different regulators at their membrane. By expressing M proteins at its surface, *S. pyogenes* efficiently recruits FH and FHL-1, diminishing its opsonophagocytosis by macrophages (Horstmann et al., 1988; Kraiczy and Würzner, 2006). In the same line, the protein Por1A from *N. gonorrhoeae* binds FH but also C4BP, allowing it to block the three complement pathways (Kraiczy and Würzner, 2006). While fluid phase regulators are easier to recruit, some pathogens have been shown to acquire membrane-bound effectors. This was observed for *E. coli* and *Helicobacter pylori*, which both can be acceptors for protectin, preventing the insertion of MAC at the cell surface thus protecting the pathogen (Rautemaa et al., 2001, 1998). Bacteria can also induce the proteolytic cleavage of key complement proteins. While the elastase B of *P. aeruginosa* (described in more details after) degrades the molecule C3 (among many others), staphylokinase from *S. aureus* induces plasmin-dependent C3b degradation at the bacterial surface thanks to the activation of the host's plasminogen (S. H. M. Rooijackers et al., 2005). *S. aureus*, which possesses a complete arsenal of virulence factors interfering with the complement system, expresses the C3-binding protein Efb, preventing generation of C3b (Lee et al., 2004). In the same line,

staphylococcal complement inhibitors are small proteins able to bind and inactivate both classical and alternative C3 convertases, thus blocking the entire cascade (Suzan H. M. Rooijackers et al., 2005).

c) Interaction between the complement system and *Pseudomonas aeruginosa*

The different mechanisms by which this particular bacterium interferes with the complement cascade are summarized in [Figure 19](#) (p.66).

i) Activation of the complement system by *Pseudomonas aeruginosa*

The interplay between complement and a pathogen is initially driven by the ability of the later to activate the system. *In vitro*, using serum, it has been shown that *P. aeruginosa* is a potent activator of the three complement pathways (Baltimore and Shedd, 1983; Davies et al., 2000; Kenawy et al., 2012; Kilpatrick and Chalmers, 2012; Kuipers et al., 2003; Lambris et al., 1982; Meshulam et al., 1982b, 1982a; Pier and Ames, 1984; Schiller, 1988). However, not all strains activate each pathway with the same efficiency. While some isolates are mainly opsonized through AP activation, others require two pathways (e.g AP and CP or AP and LP) (Schiller, 1988; Younger et al., 2003). This discrepancy between strains is still poorly understood, but might come from a differential expression or accessibility of complement-activating elements expressed by the bacteria, namely the LPS and mannose residues, which are potent activators of the CP and the LP, respectively (Bignold et al., 1991; Stang et al., 2014). Requirement for specific antibodies in complement-mediated killing/opsonization also seems to be strain dependent (Baltimore and Shedd, 1983; Nakae et al., 2008; Pier and Ames, 1984; Savoia et al., 2008; Schiller, 1988; Tanaka et al., 2010). These antibodies could be found in sera from healthy donors and CF patients, and are more likely to be efficient against non-mucoid isolates as mucoid strains are less efficiently targeted by specific immunoglobulins (Baltimore and Mitchell, 1980; Weimer et al., 2009b, 2009a).

*ii) Importance of the complement system during *Pseudomonas aeruginosa* infections*

In human clinical settings

Clinical conditions favoring *P. aeruginosa* infections are often associated with reduced complement activity. For instance, *P. aeruginosa* is a leading cause of infection in neonatal intensive care units (Jefferies et al., 2012), while the serum of children is inefficient at eliminating *P. aeruginosa* compared to the adult's one (Khan et al., 2018), probably because of reduced levels of C9 and C1q (Davis et al., 1979; Høgåsen et al., 2000; Lassiter et al., 1992). Patients with burns are often colonized by *P. aeruginosa* and are at risk for life-threatening bacteremia. In addition to physical disruption of the mechanically protective barrier of the skin, burn injuries decrease complement activity in serum (Bjornson et al., 1986, 1984; Ono et al., 1994). Blister fluids from burn injuries present very low levels of complement components, greatly diminishing the ability of phagocytic cells to destroy bacteria (Deitch, 1983; Deitch et al., 1985; Ono et al., 1994). Exudates from non-thermally induced wounds, another type of infection in which *P. aeruginosa* is often isolated, also show impaired opsonic capacity (Bridges et al., 1987). In acute and chronic lung infections, genetically-based decreased capacity to activate the LP has been associated with increased and earlier colonization by the *P. aeruginosa* (Chalmers et al., 2011a, 2011b; Kilpatrick and Chalmers, 2012). In CF lungs, despite a massive recruitment of PMNs, *P. aeruginosa* is seldom eradicated. In addition to impaired internalization of the pathogen due to its embedding within the thick mucus and biofilms, neutrophil elastase released in the airways cleaves C3bi at the surface of opsonized bacteria, and CR1 expressed on PMNs, preventing *P. aeruginosa* phagocytosis (Tosi et al., 1990).

Also, there is a growing body of evidence showing that patients treated with the monoclonal antibody Eculizumab, preventing the cleavage of the complement component C5 and used in the treatment of paroxysmal nocturnal hemoglobinuria, are at risk for *P. aeruginosa* (Kawakami et al., 2018; Patir et al., 2015; Webb et al., 2016).

Genetically-based complement deficiencies in human have been described for almost all the components of the system. However, most of these primary immunodeficiencies are extremely rare (prevalence <0.05% in the population), with each time less than 100 cases

described worldwide (Brodzki et al., 2020; Pettigrew et al., 2009; Skattum et al., 2011). The exception is the MBL deficiency (serum levels <100ng/mL), which affects ~ 5 to 15% of Caucasians, and has been associated with increased risk of respiratory tract infections when in combination with other primary or secondary immunodeficiencies (Brodzki et al., 2020; Skattum et al., 2011). However, most complement deficiencies increase the susceptibility to encapsulated pathogens like *N. meningitidis* and *S. pneumonia* (Skattum et al., 2011), but not particularly to *P. aeruginosa*. This means that other immune effectors (e.g neutrophils) need to be compromised to favor *P. aeruginosa* infections.

In animal models

In vivo, the importance of the complement system to fight *P. aeruginosa* has been studied mainly using mice models with various infection types. In a mouse model of acute pneumonia, complement depletion using cobra venom factor or genetic knockout of key components C3, C5 and C5aR, increased proliferation of the bacteria and mortality (Cerquetti et al., 1986; Gross et al., 1978; Höpken et al., 1996; Kenawy et al., 2012; Larsen et al., 1982; Mueller-Ortiz et al., 2004; Younger et al., 2003). While trying to identify which activation pathway was involved in the defense of the lung, Mueller-Ortiz and colleagues (Mueller-Ortiz et al., 2004) determined that the AP is essential, as C3 and Factor B deficient mice were highly susceptible compared to WT and C4^{-/-} animals, due to the absence of complement-mediated opsonophagocytosis rather than MAC-induced lysis. Indeed, serum from WT animals is often inefficient at killing *P. aeruginosa* by MAC formation, even strains hyper sensitive to human serum (Lin et al., 2019; Mueller-Ortiz et al., 2004; Ohno et al., 1995; Younger et al., 2003). In a burn injury model of infection, another clinical condition in which *P. aeruginosa* is a leading cause of complications, protective complement-dependent opsonophagocytosis is mediated essentially by the LP, as MBL^{-/-} mice are all killed by the bacteria while 70% of WT animals survive (Møller-Kristensen et al., 2006). Injection of soluble CR1, preventing the cleavage of C3 and C5, also increases the lethality of *P. aeruginosa* bacteremic rats (Swift et al., 1994).

It was observed that MBL^{-/-} mice were as efficient as WT to clear the bacteria in their lung (Kenawy et al., 2012), with no differences in survival. The deleterious effect of a lack of

C5aR was not observed when the infection was peritoneally induced, as opposed to a lung infection (Höpken et al., 1996). This means that the role of complement against *P. aeruginosa* might vary between organs.

Although the complement system is in overall beneficial for the host during *P. aeruginosa* infection, it is important to note that its role in the inflammatory response can be deleterious. Deletion of the anaphylatoxin receptor C3aR in mice allows the animals to better clear the bacteria, while recruitment of PMNs and cytokines' release are both decreased (Mueller-Ortiz et al., 2004). In CF patients with chronic *P. aeruginosa* lung infection, a positive correlation was observed between the complement activation and the decrease in lung function (Conway Morris et al., 2009; Pressler et al., 1995). In line with this harmful over-activation of the complement, which is often observed in the hyper-inflammatory condition of sepsis, complement depletion decreased the severity of *P. aeruginosa* induced bacteremia in a porcine model of infection (Dehring et al., 1987).

iii) Bacterial and host factors involved in the interplay between the pathogen and the complement system

Direct interference with effectors involved in complement activation

P. aeruginosa produces a whole array of proteases essential for pathogenesis (Galdino et al., 2019; Steadman et al., 1993). The metalloproteinase elastase LasB, also known as pseudolysin, degrades key components of the complement system, including C1q, C2, C3, C5, C6, C8 and C9, with C3 being the most targeted molecule, therefore greatly affecting all three complement pathways (Hong and Ghebrehiwet, 1992; Schmidtchen et al., 2003; Schultz and Miller, 1974) and reducing bacterial internalization by PMNs (Weber et al., 1982). *In vivo*, its beneficial effect during a murine lung infection model was confirmed, due to its involvement in the reduction of C3 and Factor B concentrations in bronchoalveolar lavage fluid (BALF) of infected mice (Bastaert et al., 2018). Interestingly, LasB doesn't seem to play a role during bacteremia (Wretling and Pavlovskis, 1983), likely due to the important amount in the blood of protease inhibitors α -2 macroglobulin, amyloid P-component, α 1-antitrypsin and inter- α -

inhibitor (Schmidtchen et al., 2003; Steadman et al., 1993; Vachino et al., 1988). The second major protease secreted by *P. aeruginosa* is the alkaline protease A (AprA). Like LasB, it degrades complement component C1q, C2 and C3, with C2 being the most targeted component when physiological concentration of AprA are used, while C3 can be degraded in presence of increased amount of proteases or after a prolonged incubation time (Hong and Ghebrehiwet, 1992; Laarman et al., 2012). AprA seems to be much more efficient than LasB at degrading C3 (Hong and Ghebrehiwet, 1992), but this has been observed *in vitro* using purified proteins and doesn't consider the fact that LasB could be secreted at a higher rate than AprA (Galdino et al., 2019; Schmidtchen et al., 2003), likely compensating its lower activity. AprA can be inhibited by α_1 -proteinase inhibitor (Steadman et al., 1993), which is a target of LasB, suggesting that these two proteases can have a complementary action to enhance each other activity. In addition to LasB and AprA, protease IV and the small protease PASP are able to degrade C1q and C3, and are both involved in the pathogenesis during keratitis (Engel et al., 1998; Tang et al., 2013). Like other Gram-negative pathogens, *P. aeruginosa* expresses the protease inhibitor ecotin, which very efficiently prevents the activation the three MASPs, thus limiting C3 binding and MAC insertion at the bacterial surface, enhancing the survival of the bacteria within serum (Nagy et al., 2019).

Preventing bacterial opsonization

P. aeruginosa forms biofilms thanks to the secretion of various exopolysaccharides (EPS) like alginate, Psl and Pel. Alginate is a negatively charged polymer released in high quantity by CF isolates, responsible for their mucoid phenotype. It has been shown that this exopolysaccharide prevents activation of the complement system thanks to the presence of acetate residues bound to hydroxyl groups, making them inaccessible for C3b and C4b (Pedersen et al., 1990; Pier et al., 2001). Strains unable to O-acetylate their alginate efficiently activate the AP and are well phagocytosed by purified blood leukocytes, while acetylated strains are protected from opsonophagocytosis (Pier et al., 2001). In this line, planktonic cells are better complement-activators than biofilms (Jensen et al., 1993), which are possibly hidden thanks to the alginate composing these complex structures in CF isolates.

It has also been observed that spontaneous non-mucoid revertants originating from CF strains present a decreased survival in serum than their parental strains (Hoffmann et al., 2007), further confirming that alginate is an important factor in the interplay between *P. aeruginosa* and the complement system.

Psl and Pel are neutral exopolysaccharides present at the surface of some strains and are the major components of the biofilms formed by non-CF isolates (Franklin et al., 2011; Wozniak et al., 2003). Psl reduces the binding of complement components C3, C5 and C7 at its surface in mucoid and non-mucoid strains (Jones and Wozniak, 2017; Mishra et al., 2012; Thanabalasuriar et al., 2017), thus decreasing its susceptibility to complement-mediated phagocytosis by neutrophils and macrophages *in vitro* (Mishra et al., 2012) and *in vivo* (Thanabalasuriar et al., 2017), increasing its fitness during a murine pulmonary infection (Mishra et al., 2012). Psl exerts its protective role by diminishing the accessibility of the complement C3 binding acceptor OprF present at the bacterial surface (Mishra et al., 2015). The use of PslG, a glycoside hydrolase able to degrade Psl and Pel, induced an increased opsonophagocytosis and a reduction in bacterial burden during mouse lung infection, confirming the involvement of these EPS in bacterial protection toward complement-mediated opsonization (Pesttrak et al., 2019). Survival of a mutant devoid of Psl was unaffected in serum compared to the parental strain, showing that the beneficial role of Psl has no effect on C9 insertion in the membrane (i.e MAC formation) (Mishra et al., 2012).

P. aeruginosa also diminishes the number of accessible complement receptors thanks to the two-component regulatory system PhoP/PhoQ, allowing the bacteria to sense Mg^{2+} . PhoP/PhoQ represses the expression of the complement acceptor OprH when exposed to physiological concentration of Mg^{2+} , efficiently reducing C3 binding at the pathogen surface, thus diminishing the internalization by phagocytes and increasing the bacterial pathogenesis *in vivo* (Qadi et al., 2017).

Physical impairment of MAC insertion by LPS

LPS, also known as endotoxin, is a major constituent of the envelope of *P. aeruginosa*, which role, structure and diversity have been reviewed in (Alexander and Rietschel, 2001; Lam et al., 2011) (Figure 18). Mucoid CF isolates are most often non-serotypeable as they lack the O-specific antigen (OSA) and produce so called “rough” LPS (Hancock et al., 1983). This absence of long LPS renders these strains sensitive to complement mediated-lysis in serum (Goldberg and Pier, 1996; Hancock et al., 1983; Schiller et al., 1984). LPS protects bacteria from complement by forming a physical barrier hampering the proper MAC insertion within the cell membrane, as serum resistant isolates bind less C9 than serum-sensitive bacteria (Schiller, 1988; Schiller and Joiner, 1986). In the same line, complement resistant strains bind less C3 than their sensitive counterpart, while they surprisingly activate more efficiently the CP (Schiller, 1988; Schiller et al., 1989; Schiller and Joiner, 1986). *P. aeruginosa* LPS has been shown to be a very potent activator of the CP, inducing a total consumption of this pathway while LPS from other pathogens almost didn't activate complement (Bignold et al., 1991). In addition, C3 bound at the surface of these resistant strains was found to be mainly iC3b, therefore preventing further downstream events of the complement cascade, while sensitive isolates were covered with equal amounts of C3b and iC3b (Schiller et al., 1989). Evidences that these *in vitro* observations are true also *in vivo* have been highlighted in various murine models of infection in which synthesis of long O-antigen has been associated with increased virulence during pulmonary or ocular infections and increased survival in murine blood during endogenous bacteremia accompanied by a decreased phagocytosis by Kupffer cells (Ohno et al., 1995; Priebe et al., 2004). The fact that CF isolates usually never spread and that strains with rough LPS are mainly found in CF lungs are also good indicators of the importance of LPS in acute infections (Ohno et al., 1995; Pier, 2007). In CF isolates, loss of long OSA repeats is nevertheless beneficial as it dampens the proinflammatory response usually induced by the LPS (Goldberg and Pier, 1996; Pier, 2007), while bacteria are protected from the complement as they are embedded in the thick mucus of CF airways and form biofilm-like structures rich in alginate. Growth conditions like high NaCl concentrations, temperature or pH can affect the amount of long O-antigen (Kropinski et al., 1987; McGroarty and Rivera, 1990), thus the ability of a strain to cope with the complement system will vary depending on the organ which is infected.

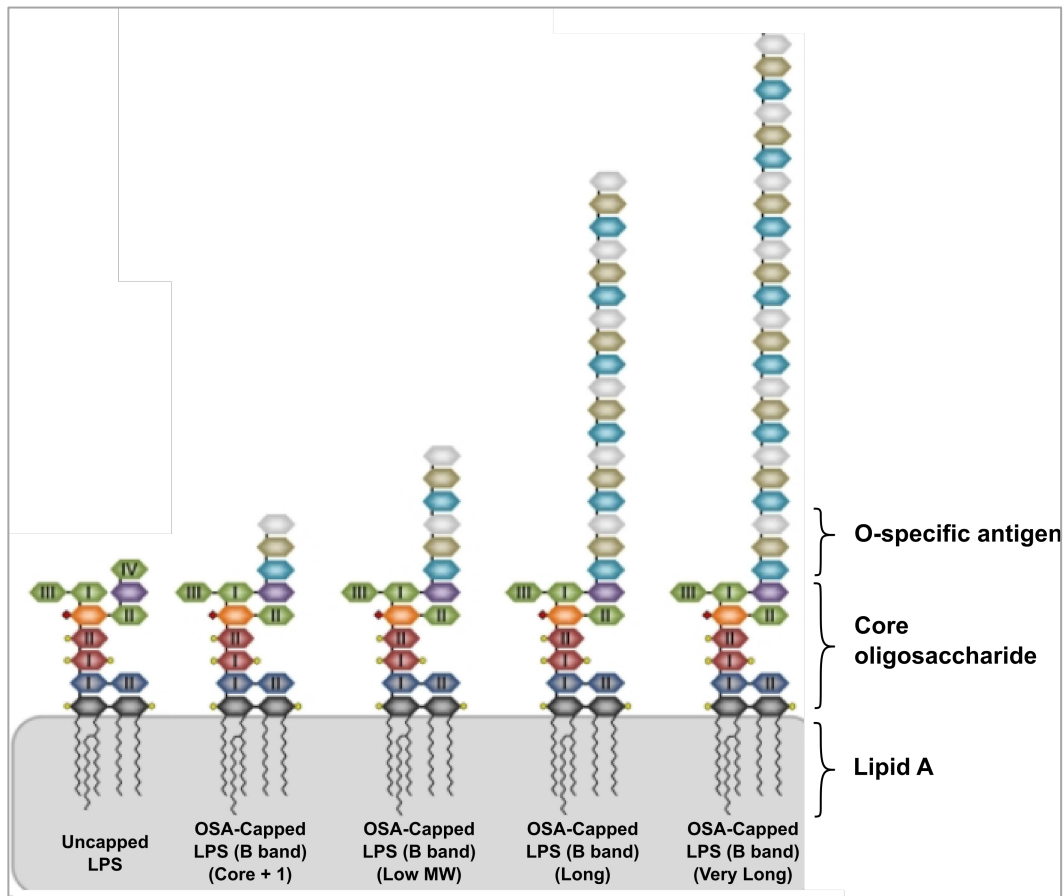


Figure 18: Structure of the LPS of *P. aeruginosa* and its different forms. Lipid A anchors the structure at the outer membrane. The core oligosaccharide represents a platform on which O-specific antigen (OSA) can be branched. OSA are heteropolymers composed of two to five different sugars, varying from strain to strain and defining the serotype of the isolate. Twenty different OSA have been described so far. If several identical polymers are assembled together, they can form long or even very long repeats, representing a physical barrier for the proper MAC insertion. The two proteins Wzz1 and Wzz2 are responsible for the control of the repeats' length, with Wzz1 generating the long form (12-30 repeats) and Wzz2 the very long form (40-50 repeats). CF isolates usually harbor an uncapped (or rough) LPS, without OSA. Adapted from (Lam et al., 2011).

Recruitment of host's complement negative regulators from the fluid phase

In addition to proteases, EPS and LPS, which directly interact with complement effectors involved in the activation of this system (e.g C1q or C3), *P. aeruginosa*, like many other pathogens, also harbors surface proteins able to bind negative complement-regulators present in the body fluids. These effectors, which could be qualified as Complement Regulator-Acquiring Surface Proteins (CRASPs) (Zipfel and Skerka, 2009), are most often moonlighting

proteins, which primary function was probably not meant to be involved in the interplay with the complement system. The translational elongation factor Tuf, delivering tRNAs to the ribosome (Harvey et al., 2019), has been identified as a partner for circulating FH and factor H-related protein-1 (FHR-1) (Kunert et al., 2007), the latter being an inhibitor of the C5 convertase. Once bound to the bacterial membrane, FH induces the conversion of C3b to inactive iC3b, preventing downstream amplification of the complement cascade. Interestingly, Meri and colleagues (Meri et al., 2013) observed that when bound to purified Tuf, the regulatory activity of FH was increased. FH depletion from the serum renders a resistant strain highly sensitive to complement mediated killing (Kunert et al., 2007), making this elongation factor a very potent virulence factor allowing the strain to withstand complement attack. However, Tuf might mainly protect from complement mediated lysis but not from opsonophagocytosis, as complement receptors CR3 and CR4 at the surface of phagocytic cells recognize iC3b (Underhill and Ozinsky, 2002). As described previously, serum-resistant strains are mainly covered with iC3b compared to the sensitive ones; it is therefore possible that these strains bind more FH at their surface. Similarly to Tuf, the dihydrolipoamide dehydrogenase Lpd, which was originally described as part of the pyruvate dehydrogenase complex within the cytoplasm (de Kok et al., 1998), can be found at the surface of *P. aeruginosa* and binds the three complement regulators FH, FHR-1 and FHL-1, conferring complement resistance to the bacterium (Hallström et al., 2012). This protein has been subsequently identified as a simultaneous partner for the two terminal pathway regulators vitronectin and clusterin, which both prevent MAC formation (Hallström et al., 2015). It is known that many pathogens hijack the physiological role of vitronectin in order to escape complement mediated lysis (Singh et al., 2010). Like with FH, vitronectin or clusterin-depleted serum was able to kill the bacteria, as opposed to normal serum (Hallström et al., 2015). Lpd can bind vitronectin and FH at the same time (Hallström et al., 2015), increasing its negative complement regulation at the bacterial surface. *In vivo*, LPS and outer membrane vesicles trigger a potent inflammatory reaction, leading to plasma exudation from blood to lungs, thus increasing the tissue concentration of vitronectin (Paulsson et al., 2018). Vitronectin from BALF of patients with pneumonia is available at a sufficient level to protect *P. aeruginosa* from complement mediated-lysis, highlighting the relevance of CRASP in the pathogenesis of this organism (Paulsson et al., 2018). The importance of Tuf and Lpd was indirectly highlighted by the observation of Dzvova and colleagues (Dzvova et al., 2017), who identified heparinase as an essential factor for *P.*

aeruginosa survival within serum. The exact mechanism by which HepP protects the bacteria was not determined, but it is possible that heparinase enables the binding of vitronectin and/or FH to Lpd and Tuf. Indeed, heparin competes with Lpd and Tuf to bind their target (Hallström et al., 2015; Meri et al., 2013). Thus, by degrading circulating heparin, HepP might facilitate the recruitment of the two complement regulators at the bacterial surface, subsequently protecting bacteria from complement mediated-lysis. It has also been noticed that the presence of the single nucleotide polymorphism rs3753394 C/T in complement factor H gene, which increases the level of FH in plasma, is significantly higher in bacteremic patient with *P. aeruginosa* (Li et al., 2020). It is possible that in these patients, bacteria are better protected from MAC insertion, as more FH will be available to bind Tuf and Lpd. By uptaking sialic acid from its surrounding environment, *P. aeruginosa* is also capable to recruit FH at its surface (Khatua et al., 2010).

Inhibitory antibodies

Recently, Wells and colleagues (Wells et al., 2014) described for the first time the presence of high concentration of seric IgG2 directed against the LPS of *P. aeruginosa* invading strain, during chronic bronchiectasis. These immunoglobulins were shown to mediate protection of the bacteria against complement-mediated killing in serum but also against phagocytosis by blood leukocytes, elucidating the ununderstood phenomenon observed by Thomassen and Demko (Thomassen and Demko, 1981), who described a ‘‘blocking factor’’ in sera of CF patients with poor clinical conditions. This unique interplay has been shown to be highly specific between the patient and its cognate strain, as the patient’s serum was effective at killing other clinical isolates while the patient’s strain was eliminated in normal human serum. Isolates coming from patients producing these inhibitory antibodies seem to bind more MAC than other clinical isolates, however, due to high quantity of long O-antigen synthesized by these strains, these complexes might not be inserted correctly within the membrane. These IgG2 are also found in the patient’s sputum, making difficult the clearance of *P. aeruginosa* within the lungs of these patients. Affecting about 20% of the chronically infected patients, this phenomenon is of high concern, but can hopefully be attenuated with the use of plasmapheresis. Indeed, removal of these specific immunoglobulins in patients has been shown to restore

complement-mediated killing of the pathogenic isolate, leading to the disappearance of *P. aeruginosa* from sputum, decreased inflammation and therefore great improvement of the patient's well-being (Wells et al., 2017). Reappearance of these antibodies 3 months following plasmapheresis correlated with reappearance of *P. aeruginosa* in the patient's sputum, requiring new steps of plasmapheresis to improve the patient's outcome. A post-lung transplant patient with life-threatening *P. aeruginosa* infection was recently cured using plasmapheresis (Divithotawela et al., 2020), showing at the same time the clinical impact of these inhibitory antibodies and the need of an effective complement system during *P. aeruginosa* infections.

Maintenance of membrane integrity

Maintenance of membrane integrity is crucial to cope with MAC mediated-lysis. This was well illustrated in the study of Munguia and colleagues (Munguia et al., 2017), in which they show that a functional Mla pathway, allowing to maintain the lipid asymmetry of the outer membrane, is needed for *P. aeruginosa* to resist elimination in human serum and whole blood. Interestingly, the same pathway was identified a few months before as being involved in serum-resistance in *A. baumannii* (Sanchez-Larrayoz et al., 2017a), highlighting the importance of keeping a proper membrane structure to deal with MAC insertion among different Gram-negative bacteria. Even though some controversies exist regarding the exact role of the Mla pathway in the transport of glycerophospholipids, several reports have shown that this system is essential to resist stresses targeting the membrane (Malinverni and Silhavy, 2009; Munguia et al., 2017; Powers and Trent, 2019).

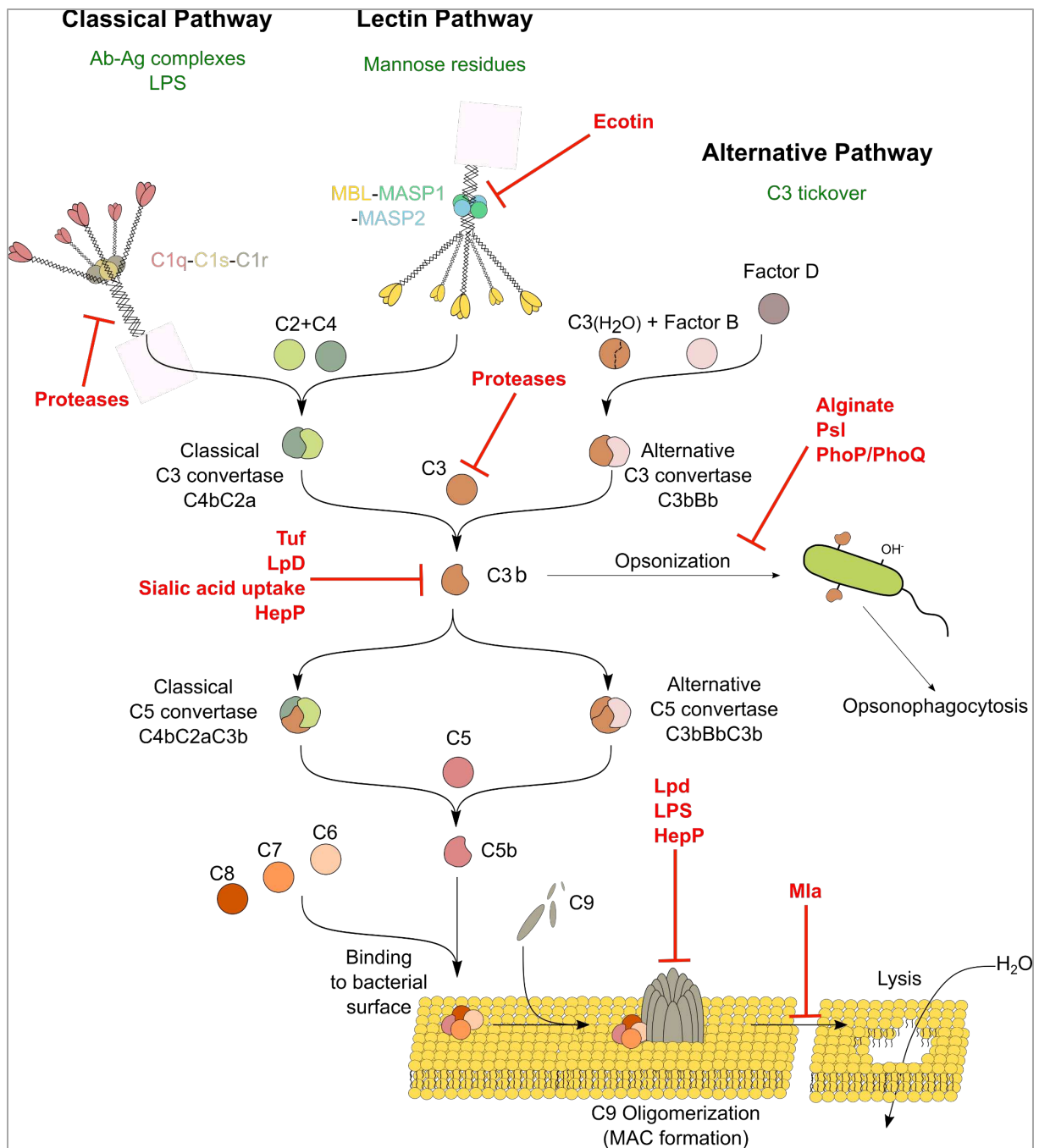


Figure 19: *P. aeruginosa* efficiently interferes with the complement cascade at various key steps. Inactivation of the CP and LP is mediated through the degradation of C1q and C3 by proteases (LasB, AprA, protease IV, PASP) or the inactivation of the MASPs by ecotin. The central component C3 is also subject to degradation by proteolytic enzymes. By preventing the binding of C3b to its acceptors at the bacterial surface, exopolysaccharides and the two component system PhoP/PhoQ prevent opsonophagocytosis by professional phagocytes. Thanks to their CRASP-related function and favored by HepP, Tuf and LpD recruit negative complement regulators at the bacterial membrane including FH, virtonectin an clusterin, either blocking the cascade by generating iC3b or preventing MAC insertion and subsequent envelope rupture. Uptake of sialic acid also contributes to inactivation of events requiring intact C3b.

Chapter III

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative bacterium firstly described by the French pharmacist Carl Gessard in 1882 (Gessard, 1984), while trying to identify the bacteria responsible for the blue coloration appearing on bandages. This unique pigmentation was attributed to its ability to secrete pyocyanin, the reason why it was initially named *Bacillus pyocyaneus*.

This mesophilic bacteria, able to grow optimally at temperatures up to 42°C, is often described as ubiquitous in our environment, as it has been isolated from a plethora of niches including soil, water, plants, animals and humans (Coggan and Wolfgang, 2012; Pirnay et al., 2005; Streeter et al., 2016; Walker et al., 2004). However, it has been recently suggested that even though *P. aeruginosa* can be found in these environments, its presence is most of the time linked with human activity (Crone et al., 2020). Indeed, environmental samples contaminated with hydrocarbons and pesticides were repeatedly positives (83%) for *P. aeruginosa*, while pristine samples were rarely (12%) associated with the presence of this bacterium.

1) Taxonomy and genetic specificities

P. aeruginosa belongs to the very diverse genus *Pseudomonas* comprising at least 128 different species, whose members have been isolated worldwide in virtually all kind of environments (Peix et al., 2009; Spiers et al., 2000). Within this genus, *P. aeruginosa* harbors the longest genome, whose first complete sequence was obtained 20 years ago using the reference strain PAO1 (Stover et al., 2000). Since then, 208 isolates have seen their genetical material completely sequenced, with genome size ranging approximately from 5.5 to 7 Mb, and a high G+C content between 65 and 67% (Klockgether et al., 2011; Stover et al., 2000). When compared to other well-known Gram-negative species, the genome of *P. aeruginosa* appears to be much bigger, thus encoding a higher number of proteins (Table 2). This characteristic is most likely at the origin of the impressive versatility of this bacterium. Indeed, *P. aeruginosa* is known to be metabolically highly versatile, able to use a whole array of organic compounds as carbon and energy sources (Stanier et al., 1966) and capable of anaerobic respiration through denitrification (Arai, 2011), allowing it to persist in harmful environments in which nutrients are scarce. Its ability to adapt its metabolism in a nutrition-dependent manner hinges on the

extremely high number of regulatory and two components systems found in its genome, representing more than 8% of the total genes (Frimmersdorf et al., 2010; Stover et al., 2000). In addition, *P. aeruginosa* carries an important diversity of transport systems (almost 200) involved in the acquisition of nutrients and small molecules from the surrounding environment. Regardless of their origin (i.e environmental vs clinical), all *P. aeruginosa* isolates share the same core genome, representing around 90% of their genes (Kung et al., 2010). The last 10%, in which the gene content might greatly vary from one strain to another, form the accessory genome. These strain-specific genes form clusters, known as regions of genomic plasticity (Mathee et al., 2008). The vast majority of the accessory genome is acquired by horizontal gene transfer from other bacterial species, and can be identified thanks to its lower G+C content compared to the rest of the chromosome (Kung et al., 2010). This accessory genome allows the bacteria to better adapt to its specific environmental niche, thus increasing its fitness (Kung et al., 2010). Another important element driving the adaptation of a strain to its environment is the presence of insertion sequences, mobile genetic elements which influence the expression of neighboring genes upon insertion, either in the positive or negative way (Sentausa et al., 2019; Siguier et al., 2014; Vandecraen et al., 2017).

Species	Median genome size (Mb)	Median protein counts	Median G+C content (%)
<i>Pseudomonas aeruginosa</i>	6.6	6113	66.2
<i>Klebsiella pneumoniae</i>	5.6 (85)	5344 (87)	57.2
<i>Escherichia coli</i>	5.1 (77)	4769 (78)	50.6
<i>Salmonella enterica</i>	4.8 (73)	4477 (73)	52.1
<i>Vibrio cholerae</i>	4.0 (61)	3574 (58)	47.5
<i>Acinetobacter baumannii</i>	4.0 (61)	3692 (60)	39

Table 2: Global genome characteristics of *P. aeruginosa* compared to other Gram-negative species. Values in () indicate the percentage compared to the values recorded for *P. aeruginosa*.

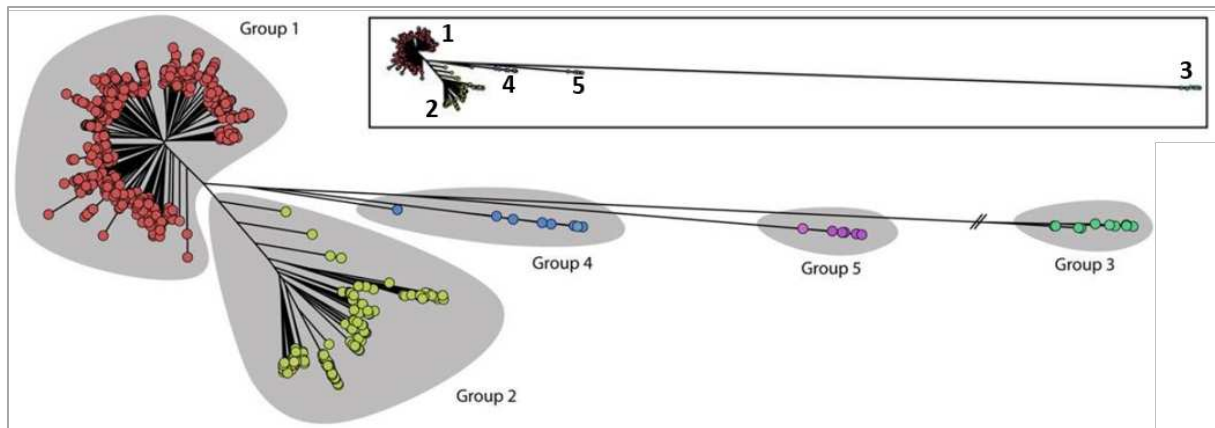


Figure 20: The diversity of *P. aeruginosa* strains. Phylogenetic analysis based on Single Nucleotides Polymorphisms (SNPs) identified in 448 core genes from 1315 isolates. The small tree in the box represents the real distance between group 3 and the four other groups. From (Freschi et al., 2019).

To date, five different groups of strains have been identified, when comparing the sequences of 448 genes of the so-called core genome (Figure 20). Each of the groups 1, 2 and 3, which are the most populated ones, present a reference strain which has been the most studied compared to the other isolates of the clade. Groups 1 and 2 are represented by the two clinical isolates PAO1 and PA14, which were isolated respectively in 1955 and 1995, from a wound and a burn infection (Holloway, 1955; Rahme et al., 1995) and are now considered as laboratory strains. They were the first *P. aeruginosa* isolates to be fully sequenced (Lee et al., 2006; Stover et al., 2000). PA7 was firstly described in 2010 (Roy et al., 2010), and became the reference strain of the group 3. Isolates from this later group are distant from the groups 1, 2 and 4, as the core genome of PA7 shares only 93.5% identity with the one of PAO1, while PA14 and PAO1 present >99% homology between their two core genomes (Roy et al., 2010). Along with an important divergence in terms of gene sequences, strains from group 3 and 5 differ from classical isolates mainly because they lack all genes coding for structural, regulatory and effector components of the T3SS (Reboud et al., 2016). This macromolecular complex is the most important virulence factor of the *P. aeruginosa* strains belonging to groups 1 and 2. Numerous studies have shown that this apparatus and injected toxins ExoU or ExoS enhances the virulence of the pathogen in various animal models of infection including acute pneumoniae (Balachandran et al., 2007; Lee et al., 2005), bacteremia (Vance et al., 2005), keratitis (Lee et al., 2003) and burn (Holder et al., 2001). It is also known that this virulence factor worsens the prognosis and the outcomes of infected patients as isolates capable of toxin secretion by the

T3SS are most of the time isolated from patients with severe infections and persist within the host (El Solh et al., 2008; Hauser et al., 2002; Roy-Burman et al., 2001; Zhuo et al., 2008). Despite the fact that PA7-like strains do not possess T3SS, they carry the genes coding for the pore forming toxin ExlA and its cognate transporter ExlB. This two-partner secretion system was first described in 2014 as being present in a hyper-virulent strain isolated from a chronic obstructive pulmonary disease (COPD) patient (Elsen et al., 2014) and was subsequently shown to be an essential player in bacterial pathogenicity. ExlA is a potent cytolytic toxin (Basso et al., 2017a), promoting bacterial dissemination and destruction of immune cells (Bouillot et al., 2017; Reboud et al., 2017). Although *P. aeruginosa* expresses an impressive array of potent virulence factors (described all along the first two chapters), this bacterium is not considered as a true pathogen. Community-acquired *P. aeruginosa* infections are therefore unusual, and involve only rare hypervirulent strains able to infect healthy individuals (Elabbadi et al., 2019; Huhulescu et al., 2011).

2) Patients at risk for *Pseudomonas aeruginosa* infections

Along with its ability to survive in various environments, *P. aeruginosa* is capable of infecting a whole array of different hosts, including plants, amoebae, nematodes, insects and mammals (Andrejko et al., 2014; Matz et al., 2008; Stevens et al., 1994; Tan et al., 1999; Walker et al., 2004). In humans, this bacterium is considered as an opportunistic pathogen, as it mainly affects compromised individuals. Most patients with CF, a genetic disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, are colonized by *P. aeruginosa* (FitzSimmons, 1993). In this autosomal recessive disease, the function of the chloride channel CFTR is reduced or even abolished depending of the mutation, leading to a lower secretion of ions by epithelial cells which express high quantities of CFTR (Ratjen et al., 2015). In the lung, this phenomenon renders the mucus thicker, which accumulates in the airways' lumen. This environment is particularly favorable for *P. aeruginosa*, which is protected from the immune defenses and multiplies, reducing lung function and in some cases leading to patient's death (Govan and Deretic, 1996). In addition to CF patients, every immunocompromised individual (e.g, neutropenic and oncological patients) is at risk for this pathogen. Because of its tropism for weak patients and its ability to survive in hospital settings,

P. aeruginosa is mainly responsible of nosocomial infections, of which it is a leading cause (Gellatly and Hancock, 2013; Kerr and Snelling, 2009), as shown in Figure 21. As previously stated, the important versatility of this pathogen allows it to be a major cause of skin and wound infection in burn patients (Holder, 1993), keratitis (Sy et al., 2012), acute pneumoniae (Gellatly and Hancock, 2013) and BSI (Viasus et al., 2020). In France, *P. aeruginosa* mainly causes urinary tract infections and pneumonia (Figure 22).

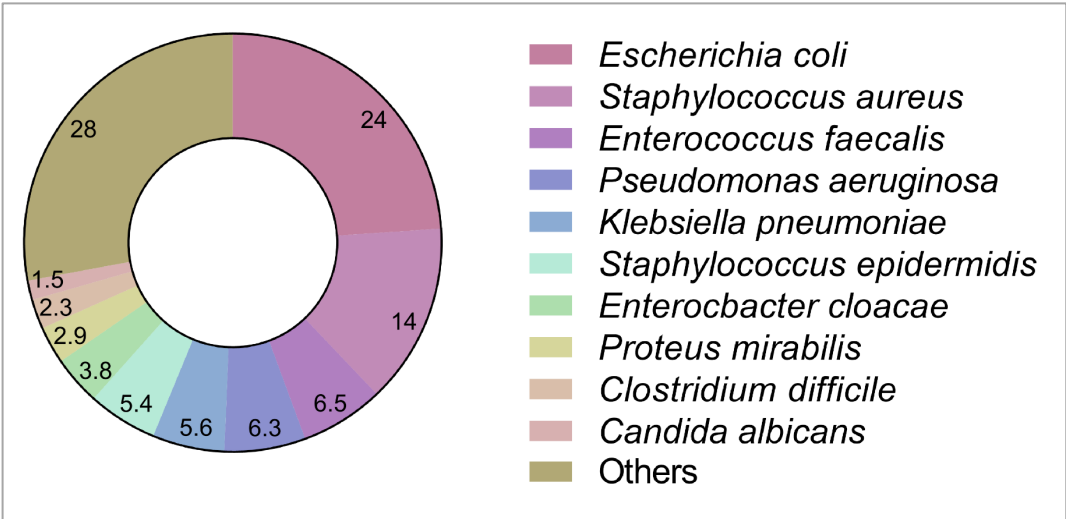


Figure 21: Prevalence of different microbial species in hospital acquired infections in France. Accounting for 6.3% of all nosocomial infections, *P. aeruginosa* is the 4th leading cause of such complication. Data are from (Santé publique France 2019, data from the period May-June 2017).

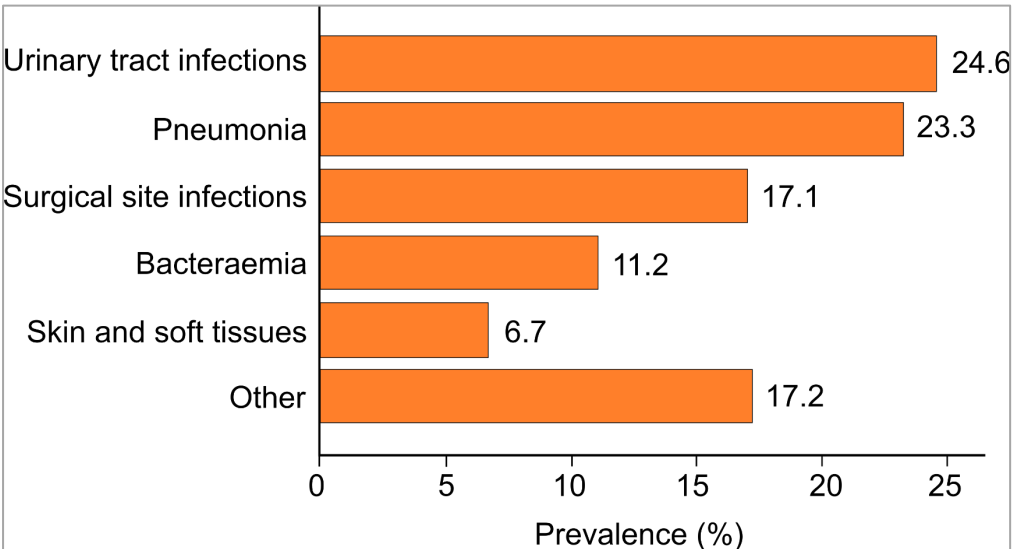


Figure 22: Principal infectious sites of hospital acquired infections caused by *P. aeruginosa*. Data are from (Santé publique France 2019, data from the period May-June 2017).

3) Acute versus chronic infections

P. aeruginosa infections can be divided in two types, acute and chronic. In the former, the bacterium evolves in a so-called “planktonic” form, invading the organism and spreading rapidly through epithelial and endothelial barriers. To do so, the pathogen expresses motility factors like the flagella or pili, but also all its arsenal of cytotoxic effectors. These types of infections are characterized by tissue damages and rapid loss of organ functions. On the other hand, bacteria can settle and multiply within an organ following a first step of colonization, giving rise to chronic infection. In this case, thanks to an important genetic reprogramming, the pathogen shifts from a planktonic toward a sessile lifestyle, allowing it to generate microcolonies in which bacterial cells reduce the expression of acute virulence factors (e.g flagella, T3SS) while they start releasing important amounts of Psl, Pel and alginates (Figure 23) (Winstanley et al., 2016). This phenomenon eventually leads to the formation of communities known as biofilms, in which bacteria are surrounded by a thick layer composed of exopolysaccharides, DNA and proteins, shielding the pathogen against the host immune defenses and antibiotics. In this context, apparition of key genetical mutations like in the genes *mutS* or *mucA*, respectively conferring a hyper-mutator and hyper-mucoid phenotype (overproduction of alginates), increase the fitness of the bacteria to persist within the organ, making them impossible to be eradicated even with the use of high doses of antibiotics (Mulcahy et al., 2010). Chronic infections are mainly observed in the lung of CF individuals but can also occur in other patients leading to chronic bronchiectasis or COPD, with high rates of mortality (Gellatly and Hancock, 2013).

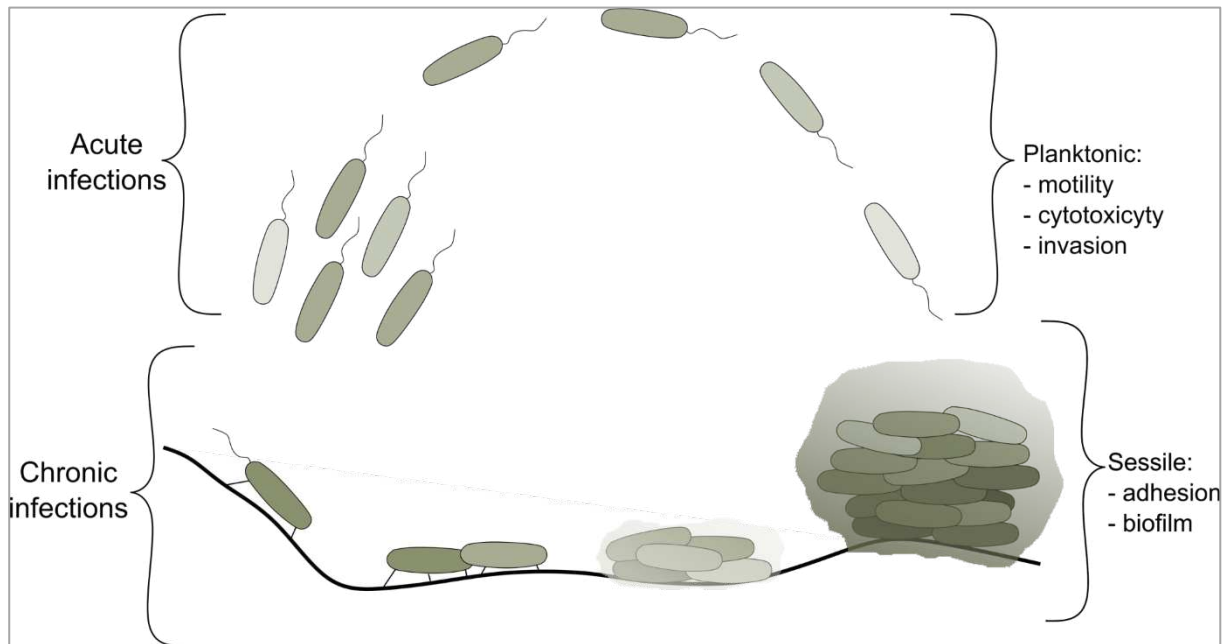


Figure 23: The different lifestyles of *P. aeruginosa* and their involvement during infection.

4) A critical concern regarding available treatments

Because of the misuse and systematic administration of antibiotics in humans but also in livestock during the last decades, the acquisition of resistances by pathogenic bacteria has been favored. As a consequence, the antibiotic pipeline is running dry while more and more bacterial strains are becoming multidrug resistant (resistant to 3 or more different classes of drugs). Recently, *P. aeruginosa* was classified by the World Health Organization as being part of the three pathogens against which there is a critical need of new antibiotics (Tacconelli et al., 2018). Indeed, the proportion of *P. aeruginosa* clinical isolates being multidrug resistant has increased by more than three times in less than ten years (Lister et al., 2009) and thus represent a major concern at the hospital, especially in ICUs (Figure 24). *P. aeruginosa* is particularly well equipped to prevent the deadly action of various classes of antibiotics, with resistance mediated by intrinsic, acquired or adaptive mechanisms. Among the intrinsic resistance, the fact that its outer membrane has a very low permeability (100 times less than *E. coli*) decreases the amount of antibiotics able to penetrate within the cell, a process essential for the proper action of various classes of molecules like quinolones, β -lactams, aminoglycosides and polymyxins (Pang et al., 2019). Efflux systems allowing the pathogen to expel intracellular

antibiotics (like MexCD-OprJ sending back β -lactams in the extracellular medium) and antibiotic-inactivating enzymes able to modify the chemical structure of potent bactericidal molecules rendering them inefficient, are the two other major intrinsic mechanisms of resistance (Pang et al., 2019). *P. aeruginosa* can become antibiotic-resistant following horizontal gene transfer during which the bacterium can acquire antibiotic resistance genes present on a plasmid carried by another cell belonging or not to the same species. Spontaneous mutations are also an important driver of resistance in *P. aeruginosa*. It has been well described that mutations preventing the function of the porin OprD is associated with increased resistance to carbapenems (Li et al., 2012). Finally, *P. aeruginosa* is also known to use adaptive mechanisms to prevent antibiotic-mediated killing. The first example is the formation of biofilms, in which the bacteria are protected as the thick exopolysaccharide layer reduces the diffusion of molecules within the structure (Ciofu and Tolker-Nielsen, 2019). Formation of persister cells, which are rare bacteria able to withstand antibiotic stress without genetic mutations, also represent an adaptive strategy and originates from phenotypic heterogeneity within the population.

Due to this major concern of antibiotic resistance, new treatments are currently being developed, based on anti-virulence strategies or using phage therapy, thought to be potent complements to antibiotic treatments.

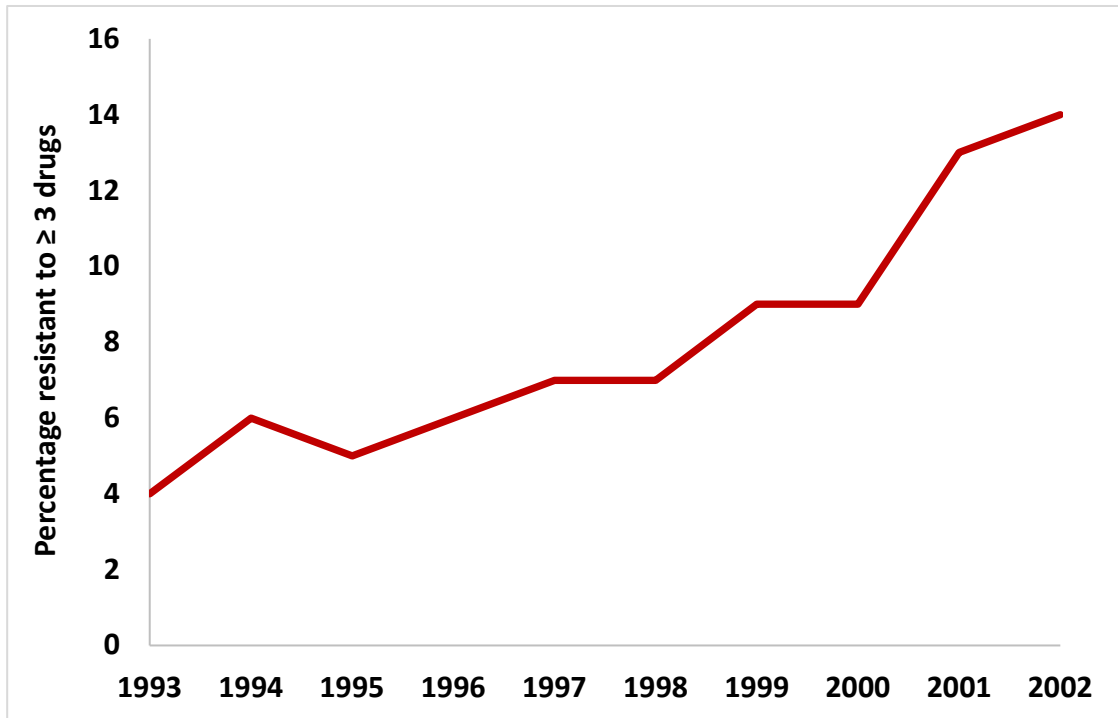


Figure 24: Evolution of the proportion of multidrug resistant isolates of *P. aeruginosa* from ICUs in the United States. Data are from (Lister et al., 2009).

Chapter IV

Phenotypic heterogeneity

1) Generalities

For a long time, it has been thought that within a bacterial population composed of genetically identical individuals, each cell would behave in the same manner when facing a stress or a new environment. However, it is now clear that gene expression is far from homogenous even in a clonal population. First, in the environment (e.g water, soil) or within the host upon infection, bacteria encounter a plethora of micro-environments (even within immune cells), with fluctuating levels of nutrients, oxygen and differences in pH. For instance, the lumen of the intestinal track is rather anoxic while the oxygen level rises at the surface of enterocytes (Marteyn et al., 2010). As each of these factors represent potent stimuli sensed by bacteria, it is easy to conceive that each individual will induce different genetic responses to evolve within these specific niches. In addition to differential gene expression triggered by the diversity of microenvironments encountered by cells even spatially close, the fact that bacteria can face sudden stresses (e.g temperature, immune response, antibiotics), also drives the appearance of divergent genetic reprogramming. In both cases, phenotypic heterogeneity arises, making population-level studies sometimes irrelevant as they monitor the sum of potentially divergent phenomenon (Weigel and Dersch, 2018).

2) Phenomena contributing to phenotypic diversity

In a homogeneous environment, (even though this might exist mainly in the lab), phenotypic heterogeneity can be triggered by different events. Asymmetric division, leading to unequal repartition of the cellular content between the two daughter cells, has been linked with phenotypic heterogeneity in *Mycobacterium tuberculosis* (Kieser and Rubin, 2014). Stochastic fluctuation, or noise, in gene expression due to the fact that important regulatory molecules are present in low numbers within cells, is an important driver of diversity in an isogenic population (Ackermann, 2015; Elowitz et al., 2002). Another mechanism leading to phenotypic heterogeneity is the process of phase-variation. Various genes are phase variable, forming the ‘phasome’, meaning that their expression can switch between an ON and OFF state, in a rapid and reversible way (Phillips et al., 2019). Regarding the molecular mechanism underlying this phenomenon, promoter inversion (among others like the presence of hypermutable DNA

sequences) generates frequent events of phase variation (Jiang et al., 2019). Invertible promoters are flanked by inverted repeats recognized by a dedicated enzyme able to invert the orientation of the promoter which is no longer able to activate the transcription of its normally cognate genes. Once inverted, the promoter in its OFF state can return to its initial ON position through the same mechanism, thus allowing the transcription of its associated downstream genes (Figure 25). Even though a region of DNA is rearranged during inversion, this process is fully reversible. Epigenetic modifications, like DNA methylation, can also induce bistable switches in gene expression (Dubnau and Losick, 2006; Phillips et al., 2019).

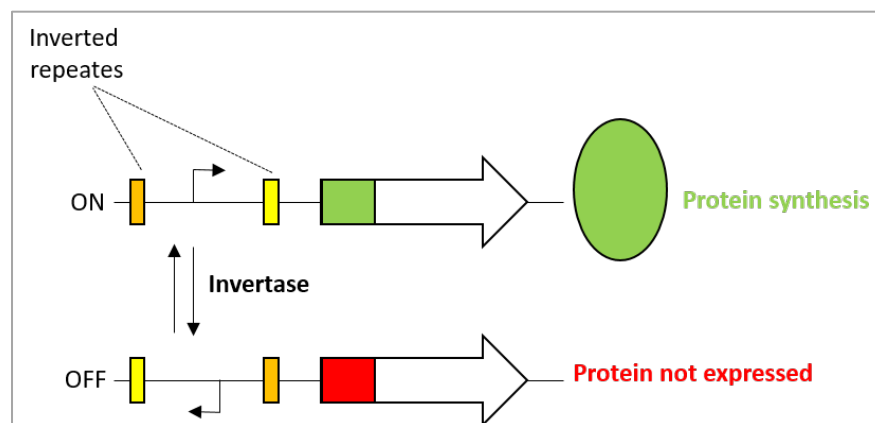


Figure 25: Phase variation mediated by invertible promoters following the action of a dedicated integrase.

3) Benefits for the bacteria

a) When facing host's immune defenses

Differential gene expression usually does not affect housekeeping functions, but rather genes involved in metabolic pathways or virulence factors which essentiality is restricted to certain specific conditions. Phenotypic heterogeneity allows a subset of cells to be better prepared to survive within a stressful environment, thus potentially ensuring that at least a part of the population survives upon a sudden stress (Reyes Ruiz et al., 2020). The fact that presence of invertible promoters in the genome of bacteria strongly correlates with their ability to colonize a host indicates that phenotypic heterogeneity increases their fitness in this context (Jiang et al., 2019). By being better adapted to a specific challenge, already before it has been

encountered, cells respond much rapidly compared to their divergent relatives. Indeed, the process of sensing and respond to an evolving environment takes time, representing a threat if the cell is not able to adapt in time. This strategy, which is beneficial only for a subpopulation, is known as ‘bet-hedging’. However, the favored subpopulation might not be selected over time in rapidly evolving conditions. This can be well conceived in the course of host infection, during which the requirement for virulence factor expression fluctuates. For instance, expression of adhesion molecules will be beneficial at the onset of the invasion to adhere on epithelial cells, but might be later recognized by the immune system (Phillips et al., 2019). Following adhesion, individuals expressing higher levels of cytotoxic effectors would be selected to spread within the tissues and deal with leukocytes recruited at the pathogen entry site. Phenotypic heterogeneity is highly valuable for microorganisms able to colonize a wide range of niches, like *P. aeruginosa*, as they will encounter a plethora of varying microenvironments. Another strategy relying on phenotypic heterogeneity, but beneficial for the entire bacterial community, is the concept of ‘division of labor’ (Zhang et al., 2016). In this case, the different subpopulations found in the community achieve specific tasks beneficial for each other. For pathogens, this process may facilitate the colonization of the host, as it can be costly for a single bacterium to express all the required factors to face the immune system and multiply. This co-operation was described in the case of *P. aeruginosa* biofilm formation. Upon contact with a surface, the Wsp system senses it before attachment and induces the production of the secondary messenger c-di-GMP, responsible of the shift from a planktonic toward a sessile lifestyle. It was shown that the activity of the Wsp system is heterogeneous within the population, triggering phenotypic heterogeneity due to differences in c-di-GMP content within two subpopulations (Armbruster et al., 2019). This phenomenon generates two distinct populations, one which adhere tightly to the surface and produces large amount of exopolysaccharides Psl and Pel contributing to the formation of the biofilm structure (the ‘founders’), while the second subpopulation remains motile and contributes to surface exploration and spread of the biofilm (the ‘the explorers’). Both populations were shown to be required for the proper development of a sessile community. Biofilms are very heterogeneous structures composed of many niches with varying pH, levels of nutrients and oxygen, further favoring the appearance of divergent subpopulations. The benefit of phenotypic heterogeneity was clearly demonstrated in the virulence of several pathogens. In *Yersinia pseudotuberculosis*, bistability in the expression of the major virulence regulator RovA

generates two subpopulations (invasive and non-invasive), essential during intestinal colonization in mice (Nuss et al., 2016; Weigel and Dersch, 2018). In *A. baumannii*, appearance of hyper-virulent variants producing a thicker capsule allows the pathogen to withstand antimicrobials from the host and disseminate within the organism (Chin et al., 2018). Interestingly, cells belonging to this subpopulation were systematically isolated from the blood of patients with BSI. In various pathogenic bacterial strains, small colony variants (SCVs) have been described to arise from clonal populations experiencing stressful conditions. These cells are characterized by their slow growth rate (around nine times lower than “normal” bacteria), reduced virulence but increased antibiotic tolerance. This is why SCVs are a major clinical concern as they promote the development of persistent infections hard to eradicate (Johns et al., 2015). This phenotype can be transmitted to the cell’s progeny, but SCVs can also revert to a WT phenotype upon stress removal. In *S. aureus*, SCVs persist within cells, probably thanks to their low production of α -toxin, allowing them to escape the immune system (Melter and Radojević, 2010). In *P. aeruginosa*, rugose SCVs were shown to trigger a higher inflammatory response than the WT with increased recruitment of PMNs at the site of infection. However, these hyper-biofilm forming cells are resistant to neutrophil phagocytosis and bactericidal products, allowing them to persist within tissues (Pesttrak et al., 2018).

Cross-feeding, another type of co-operation between bacterial cells, is likely to take place within an isogenic population. It was hypothesized that due to heterogeneity in the expression of genes involved in metabolism, *E. coli* fed with glucose generates a subpopulation of cells able to use glucose to produce acetate following glycolysis, a metabolite specifically used by a second subpopulation (Nikolic et al., 2013). In this case, one subpopulation feeds the other, but more cooperative ways of cross-feeding can be considered (Figure 26). A less cooperative type of phenotypic heterogeneity is depicted by the existence of “cheaters”. When costly bacterial products are needed, like exopolysaccharides involved in biofilm formation or siderophores allowing bacteria to acquire iron from the environment, cheaters are prone to appear (Griffin et al., 2004; Martin et al., 2020). These variants do not produce those molecules, but benefit from the ones released by their relatives. In *P. aeruginosa*, expression of T3SS genes was found to be bistable, with only a part of the population able to activate the transcription of effector genes under *in vitro* inducing conditions (Diaz et al., 2011; Hornef et al., 2000). Regarding the energy required to activate this macromolecular machinery, cells which do not

transcribe those genes might be considered as cheaters protected by the rest of the population, however these swindlers might not survive if engulfed by professional phagocytes (Heimer et al., 2013; Kroken et al., 2018).

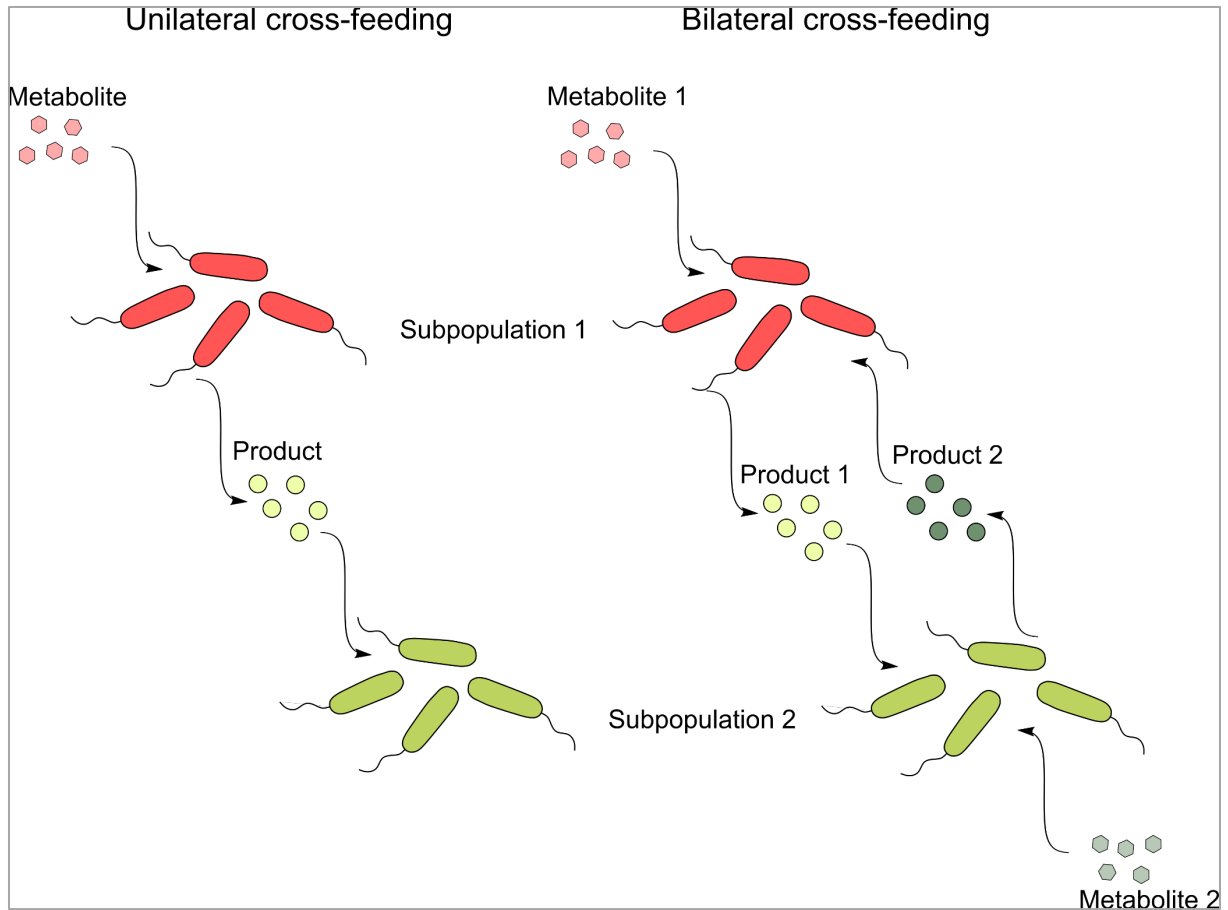


Figure 26: Cross-feeding as a potential division of labor. Phenotypic heterogeneity in metabolism allows a bacterial subpopulation to generate products used by another one. This process can be unilateral, when the subpopulation n°2 solely use the product generated by the first subpopulation. In its bilateral form, both subpopulations breakdown a specific metabolite, generating a product used by the other.

b) Antibiotic persisters

In addition to the fact that phenotypic heterogeneity enhances the ability of a population (or part of it) to withstand the host immune defenses, its involvement in the phenomenon of antibiotic persistence has been much studied since the last decade. Persisters refer to a minor subpopulation of bacterial cells able to tolerate antibiotic, while the majority of their relatives are rapidly destroyed during the treatment. When challenged by an antibiotic, microbial

populations can display four different behaviors: susceptibility, tolerance, resistance and persistence (Figure 27). Persisters started to be extensively studied when methods allowing to perform single-cell analysis began to be developed (Balaban et al., 2004). The characteristic of persister cells is that their tolerance does not originate from genetic mutations (as opposed to the appearance of resistance), rendering their phenotype transient and not transmittable to their progeny. Indeed, when the stress is over, these phenotypic variants give rise to a population as sensitive as the parental one (Brauner et al., 2016). Slow growth/dormancy seems to be the main mechanism by which persisters withstand antibiotic challenge, with the hypothesis that the drug's targets are mainly inactive in these latent cells, even though it has been observed that persisters in presence of ofloxacin do not present any growth defects compared to the rest of the sensitive population (Goormaghtigh and Van Melderen, 2019). Thus, persisters might differ between bacterial species or regarding the antibiotic used for the challenge. The molecular mechanisms by which this subpopulation is generated have been subjected to intense debates, and is still unclear. Stochastic variation in the expression of genes coding for toxin/antitoxin systems were initially propose, but has been recently refuted (Goormaghtigh et al., 2018). Induction of the stringent response due to the production of the alarmone (p)ppGpp or depletion of the pool of intracellular ATP upon entrance in stationary phase are two models accepted to explain the formation of persisters (Conlon et al., 2016; Peyrusson et al., 2020), but their involvement might differ depending of the challenge conditions (e.g if the bacteria are within phagocytes or in the external medium). Persistence can be "triggered", meaning that a signal is required to generate persisters (e.g starvation), but also "spontaneous", in which antibiotic-tolerant individuals are generated at a low but constant rate while the population has not been challenged by any stress (e.g cells entering the stationary phase stochastically in an exponentially growing culture) (Balaban et al., 2019).

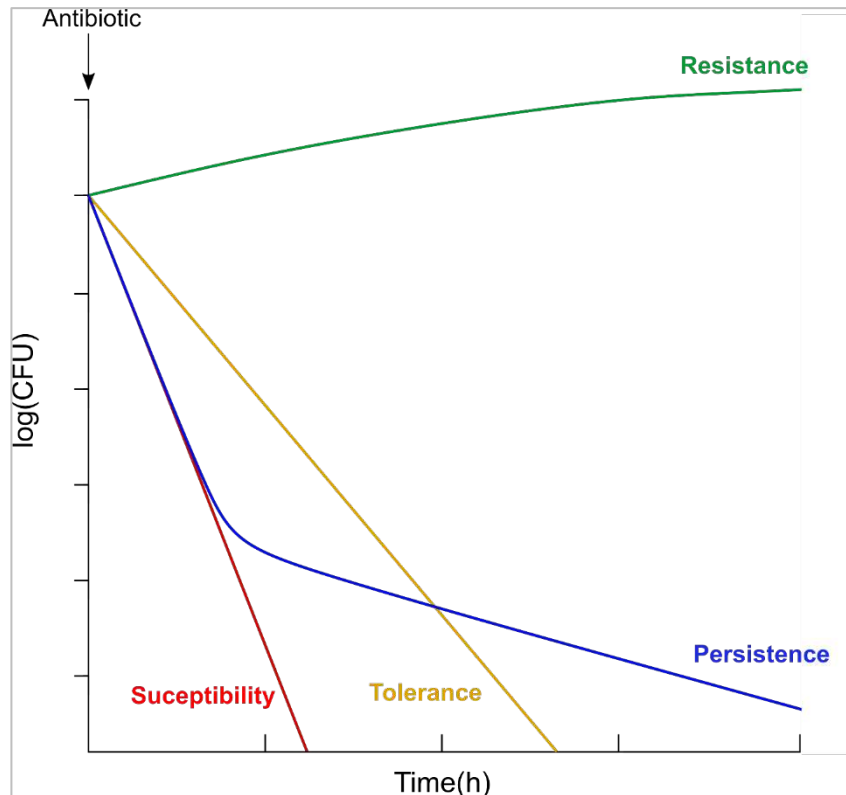


Figure 27: Differences between susceptibility, tolerance, resistance and persistence upon antibiotic stress. Susceptibility is defined as a rapid and total elimination of the entire population, while a tolerant community is also completely killed, however at a slower rate. If the population is resistant, the challenge does not influence its survival. Persistence is characterized by a typical biphasic killing curve, during which most of the population is rapidly eliminated (1st phase), leaving a minor population of tolerant cells virtually never completely eliminated (2nd phase).

Persistence contributes to the treatment failure in many types of infections, as persister cells arise within infected organs and when in contact with the immune system of the host. In CF patients, during chronic long-lasting infections with *P. aeruginosa*, late isolates are documented to generate 100 times more antibiotic persisters than early isolates (Mulcahy et al., 2010). Phagocytes, by internalizing pathogens, trigger the appearance of intracellular persisters of at least *Salmonella enterica* and *S. aureus*, due to the stressful conditions (e.g acidification) encountered upon internalization (Helaine et al., 2014; Peyrusson et al., 2020; Stapels et al., 2018). For both pathogens, intramacrophagic persisters were shown to be non-growing but metabolically active (Helaine et al., 2014; Peyrusson et al., 2020). In *Salmonella*, it was observed that toxin-antitoxin modules were required for the formation of these antibiotic-tolerant cells (Helaine et al., 2014). Using a dual RNA-Seq approach, Stapels and colleagues

(Stapels et al., 2018) identified that thanks to an active SPI-2 T3SS apparatus, *Salmonella* intracellular persisters were able to induce polarization of proinflammatory M1 macrophages toward the anti-inflammatory M2 state, meaning that these cells can manipulate the host immune response. In *S. aureus*, RNA-Seq on sorted intramacrophagic persisters revealed that these bacteria undergo a global transcriptomic reprogramming, following induction of the SOS, heat shock, cell wall stress and stringent responses (Peyrusson et al., 2020). Finally, contact with human serum was also shown to trigger the emergence of persisters in *Vibrio vulnificus*, partly due to complement activity (Ayrapetyan et al., 2015).

Results

Chapter V

Bacterial behavior in human
blood reveals complement
evaders with persister-like
features

1) Research Article

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P. aeruginosa is a leading cause of bloodstream infections, which are associated with increased death compare to other pathogens. However, despite these worrying facts, the interplay between this bacterium and the innate immune system from the circulation has been rarely studied in the complex but physiological environment of human whole blood.

This manuscript describes the behavior of *P. aeruginosa* and some other bacterial species in human whole blood and plasma. We were able to identify that rather than cytotoxicity toward circulating leukocytes, complement-resistance is the main driver of survival in human whole blood, and that the nature of the major virulence factor expressed by the strains does not affect their behavior in this context. Nonetheless, we discovered that except for a non-pathogenic strain of *E. coli*, all complement-sensitive strains were never fully eliminated; indeed we evidenced a minor subpopulation of “evaders”, able to withstand complement-mediated lysis. These rare cells, which represent sometimes less than 0.1% of the initial population, harbor a transient persister-like phenotype, however with some differences regarding the mechanisms underlying their generation. This phenomenon was observed for other bacterial pathogens, and likely represent an efficient strategy for complement-sensitive bacteria to persist within the blood and disseminate through the host.

In this work, Nathan Fraikin monitored the regrowth of complement evaders and Yvan Caspar performed the serotyping of all *P. aeruginosa* strains. I performed the other experiments.

Abstract

Bacterial bloodstream infections (BSI) are a major health concern and can cause up to 40% mortality. *Pseudomonas aeruginosa* BSI is often of nosocomial origin and is associated with a particularly poor prognosis. The mechanism of bacterial persistence in blood is still largely unknown. Here, we analyzed the behavior of a cohort of clinical and laboratory *Pseudomonas aeruginosa* strains in human blood. In this specific environment, complement was the main defensive mechanism, acting either by direct bacterial lysis or by opsonophagocytosis, which required recognition by immune cells. We found highly variable survival rates for different strains in blood, whatever their origin, serotype, or the nature of their secreted toxins (ExoS, ExoU or ExlA) and despite their detection by immune cells. We identified and characterized a complement-tolerant subpopulation of bacterial cells that we named “evaders”. Evaders shared some features with bacterial persisters, which tolerate antibiotic treatment. Notably, in bi-phasic killing curves, the evaders represented 0.1-0.001% of the initial bacterial load and displayed transient tolerance. However, the evaders are not dormant and require active metabolism to persist in blood. We detected the evaders for five other major human pathogens: *Acinetobacter baumannii*, *Burkholderia multivorans*, enteroaggregative *Escherichia coli*, *Klebsiella pneumoniae*, and *Yersinia enterocolitica*. Thus, the evaders could allow the pathogen to persist within the bloodstream, and may be the cause of fatal bacteremia or dissemination, in particular in the absence of effective antibiotic treatments.

Author summary for “Complement evaders”

Blood infections by antibiotic resistant bacteria, notably *Pseudomonas aeruginosa*, are major concerns in hospital settings. The complex interplay between *P. aeruginosa* and the innate immune system in the context of human blood is still poorly understood. By studying the behavior of various *P. aeruginosa* strains in human whole blood and plasma, we showed that bacterial strains display different rate of tolerance to the complement system. Despite the complement microbicide activity, most bacteria withstand elimination through phenotypic heterogeneity creating a tiny (<0.1%) subpopulation of transiently tolerant evaders able to persist in plasma. This phenotypic heterogeneity thus prevents total elimination of the pathogen from the circulation, and represents a new strategy to disseminate within the organism.

Introduction

The incidence of bacterial bloodstream infections (BSI) in high-income countries is as extensive as that of strokes, ranging from 113 to 204 cases per 100,000 inhabitants [1]. BSI, whether nosocomial or community-acquired, have poor prognosis, with mortality rates up to 40% [1,2]. They are also a leading cause of healthcare-associated infections in intensive care units (ICUs) [3] and neonatal wards [4], and are particularly prevalent in elderly patients (6,000 cases per 100,000 population). In this demographic, mortality rates can be up to 70% [5]. Their extensive impact on overall hospital costs (>\$40,000 per patient in the US) [6] make BSI a major public health concern.

Many different bacterial species can cause BSI, among which *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella* species, *Pseudomonas aeruginosa*, *enterococci*, *streptococci* and coagulase-negative *staphylococci* are the most prominent [1]. *P. aeruginosa* is mainly associated with nosocomial infections, and bacteremia caused by this pathogen has a poor prognosis [2,7,8] with very high mortality rates [9]. *P. aeruginosa* can survive in many different environments and colonizes plants, animals, and humans [10]. In addition to BSI, it is responsible for a number of life-threatening complications including acute pneumonia and skin infection in immunocompromised and elderly patients, as well as degradation of lung function in chronically-infected cystic fibrosis patients [11]. The major health concerns related to *P. aeruginosa* are linked to intrinsic and acquired resistance to currently available antibiotics [12].

The capacity of *P. aeruginosa* to survive in the human body hinges on a balance between its numerous virulence factors and the presence of multiple host-defense mechanisms. Regardless of the primary site of infection, *P. aeruginosa* can cross the epithelial and endothelial barriers to reach the bloodstream [13,14]. In the blood, the bacteria encounter the innate immune system, composed essentially of neutrophils, monocytes, and the complement system. Interactions between *P. aeruginosa* and this innate immune system have mainly been studied using selected strains and purified components, such as isolated complement proteins or phagocytes, or serum [15–20], in conditions differing from those found in the human blood [21,22]. Recent data indicated that systemic *P. aeruginosa* infection could lead to pathogen transmission as the bacteria were found to disseminate and propagate through the gallbladder and intestinal tract in a murine model of infection [23]. However, the mechanisms allowing bacteria to persist in the blood remained unclear. Within this study, we examined the behavior of a number of laboratory and recently isolated clinical *P. aeruginosa* strains in a standardized assay using fresh whole blood from healthy donors. Our results showed that, although complement exerts an essential antibacterial activity in the blood, individual bacterial strains display variable levels of tolerance. We evidenced, even for the most sensitive strains, the characteristic biphasic killing curves reminiscent of antibiotic persisters, and characterized a small subpopulation of phenotypic variants that we named complement evaders. These rare cells withstand complement-mediated lysis through phenotypic heterogeneity. Moreover, we discovered that several other major Gram-negative human pathogens shared the same capacity to escape human complement by forming intrinsically plasma-resistant evaders. The complement evaders may have a very significant impact on bacterial dissemination.

Results

Laboratory and clinical strains display diverse survival rates in human blood

Using a highly standardized method in human whole blood (HWB), we examined the survival of six *P. aeruginosa* strains with distinct toxin repertoires and serotypes (Fig 1). These assays included the commonly used laboratory strains PAO1 [24], PA14 [25] and PA7 [26], which belong to distinct phylogenetic lineages/groups. PAO1 and PA14 both possess the type III secretion system (T3SS) which they use to translocate ExoS or ExoU, respectively, into target host cells. PA7 lacks T3SS genes, but encodes the pore-forming toxin Exolysin A [26,27]. In addition to these laboratory strains, we included in the survey three *P. aeruginosa* strains recently isolated from infected patients [27–29]. *E. coli* CF7968, a derivative of the K12 laboratory strain [30] was added as a control (Table S1). Bacteria were incubated for 3 h in HWB from healthy donors, and bacterial survival was assessed in ten independent experiments by counting colony-forming units (CFU) (Fig 1A). Strains showed clearly distinguishable and reproducible survival rates. Approximately 10% of the laboratory and reference strains, PAO1 (ExoS⁺) and PA7 (ExlA⁺), survived, whereas only around 1% of the most sensitive strain, PA14 (ExoU⁺), was still present following the 3-h incubation in HWB. Very different survival rates were measured for the three recent clinical strains, YIK (ExoU⁺), CLJ1 and IHMA87 (ExlA⁺), ranging from 0.05% for CLJ1 to complete tolerance for YIK. Only the non-pathogenic laboratory strain *E. coli* CF7968 was completely eliminated, with no detectable CFU after 3 h exposure to HWB. The nature of the strain's virulence factors (T3SS versus ExlA) did not appear to confer any significant benefit for survival, as similar sensitivities were measured for ExlA⁺ strains and T3SS⁺ strains (e.g. IHMA87 versus PA14, or PA7 versus PAO1). In addition, survival in HWB did not correlate with a given serotype, as highly variable survival rates were measured for the three O12 strains (Fig 1B). Lack of the O antigen was detrimental for the bacteria, as illustrated by the hypersensitivity of *E. coli* CF7968.

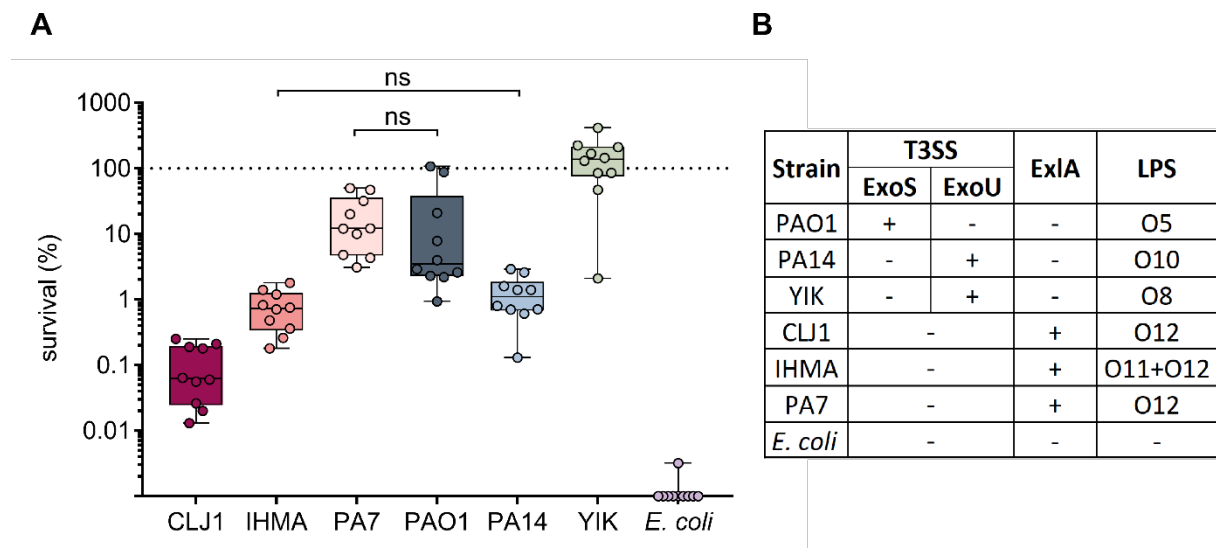


Fig1. *P. aeruginosa* strains display various rates of resilience in the HWB model. (A) *P. aeruginosa* survival in blood is independent of strain origin or the secreted toxin profile (ExoU, ExoS, or ExlA). Exponentially-growing bacteria were incubated for 3 h in blood obtained from 10 different healthy donors (n=10), and bacterial survival was determined by serial dilutions and CFU counting. ns: non-significant. When not stated, differences in survival were significant. Kruskal-Wallis test, $p < 0.001$; Student-Newman-Keuls post-hoc test: $p < 0.05$. Dots

reported on the x-axis correspond to no detectable colonies. **(B)** Nature of the toxins secreted by each strain, and serotypes of these bacteria. Note that the IHMA87 strain cross-reacts with the two antisera O11 and O12, while *E. coli* CF7968 lacks O antigens.

Intrinsic *P. aeruginosa* tolerance in blood is linked to complement activity, through either MAC insertion or opsonophagocytosis

To explore the origin of the extensive differences in survival measured in HWB, we first determined how well each strain was recognized by immune cells. While YIK induced marginal TNF α and IL6 production, all the other strains tested triggered similar high levels of cytokines (Fig S1). The toxins ExoS, ExoU, and Exolysin A are known to induce apoptosis or necrosis in a variety of eukaryotic cells, including white blood cells which may play a role in bacterial clearance from the blood [16,31–34]. We therefore examined the cytotoxic potential of each strain toward purified circulating leukocytes by monitoring lactate dehydrogenase (LDH) release. YIK induced a complete elimination of neutrophils, whereas most other strains showed similar levels of cytotoxic potential with no significant differences (Fig S2A). Every tested strain appears more efficient at killing neutrophils than mononucleated cells (Fig S2B). Thus, except for YIK, the extent to which the bacterial strains tested here were recognized by and destroyed circulating leukocytes could not explain the different survival rates measured in HWB.

We next assessed the capacity of the strains to cope with bactericidal effectors present in plasma (Fig 2A). As with bacterial survival in HWB, in plasma the survival rates for the six selected *P. aeruginosa* strains revealed a range of sensitivities from resistance to almost complete eradication. In agreement with previous reports [17,35,36], the two laboratory strains PAO1 and PA14 had contrasting survival patterns: PAO1 was tolerant, whereas PA14 was sensitive. The strains that were most tolerant to HWB (PA7, PAO1 and YIK) were fully resistant to plasma-mediated killing. The similar survival profiles between HWB and plasma, and the fact that survival of the plasma-sensitive strains CLJ1, IHMA87, and PA14 was 10-fold lower in plasma than in whole blood suggest an important role for humoral immune effectors in bacterial clearance within HWB. Due to its complete resistance to killing, the YIK strain was excluded from subsequent experiments.

To assess how fluid-phase effectors contribute to bacterial elimination in HWB, we examined the survival of the different strains by counting CFUs following incubation in HWB reconstituted after heat-inactivation of the plasma (see Material and Methods). Heat-inactivation, which eradicates complement activity, resulted in full survival of all strains, including the sensitive *E. coli* CF7968 (Fig 2B). Indeed, most strains multiplied when incubated in blood with heat-inactivated plasma, as observed by the increased CFUs compared to the starting population.

As heat-inactivation of plasma may inhibit different bactericidal plasmatic components [37], and to address whether the elimination of sensitive strains in HWB specifically relies on the complement system or other circulating effectors with antimicrobial properties, we used specific complement inhibitor OmCI (also known as Coversine) [38]. OmCI inhibits the cleavage of C5, hampering membrane attack complex (MAC) formation and subsequent complement-mediated lysis [37]. Addition of OmCI in plasma resulted in complete rescue of *P. aeruginosa* strains CLJ1, IHMA87 and PA14 from killing, demonstrating that the *P. aeruginosa* elimination in HWB is solely dependent on complement microbicide activity (Fig 2C).

The antimicrobial activity of complement relies on three main mechanisms: i/ bacterial lysis due to the insertion of the MAC into the bacterial envelope [39], ii/ opsonophagocytosis, which combines C3b binding at the pathogen's surface and its recognition by complement receptors [39], and iii/ formation of neutrophil extracellular traps (NETs) to trap and kill invading bacteria [40]. To elucidate which of these mechanisms was involved in bacterial clearance, we treated the blood with Cytochalasin D to inactivate phagocytosis, or with DNase I to prevent NETs formation [22,41] and monitored bacterial survival. Following treatment with DNase I, the same level of bacterial elimination was observed as with untreated HWB (Fig 2D), indicating that NETs play a negligible role in the process observed here. In contrast, PA7 and PAO1 elimination appear to involve some internalization by phagocytes, as their survival was consistently increased in Cytochalasin D-treated blood (Fig 2D). Because the clearance of these two strains was also complement-dependent, we conclude that they are eliminated through opsonophagocytosis. Blocking phagocytosis had no impact on the other complement-sensitive strains CLJ1, IHMA87, PA14, and *E. coli* CF7968, suggesting that they are killed through direct MAC-induced lysis.

Importantly, even though most of the strains were highly sensitive to complement, we recurrently detected a subpopulation of survivors corresponding to <1% or even 0.002% of the initial inoculum, in HWB and plasma, respectively (Fig 1A and 2A). These results suggest that a small bacterial subpopulation, that we termed "evaders", differ in phenotype from the majority of the population, and display increased tolerance to complement-mediated killing. The level of residual surviving cells appears higher (by 1-log) in whole blood than in plasma (Fig 1A and 2A). This higher clearance of bacteria in plasma than in HWB was already observed in the case of *Salmonella typhimurium* [42]. A possible explanation could be that within HWB, complement activity is more strongly repressed due to all membrane-bound negative regulators at the surface of circulating leukocytes (i.e CD59, CR1, DAF), while absent in plasma.

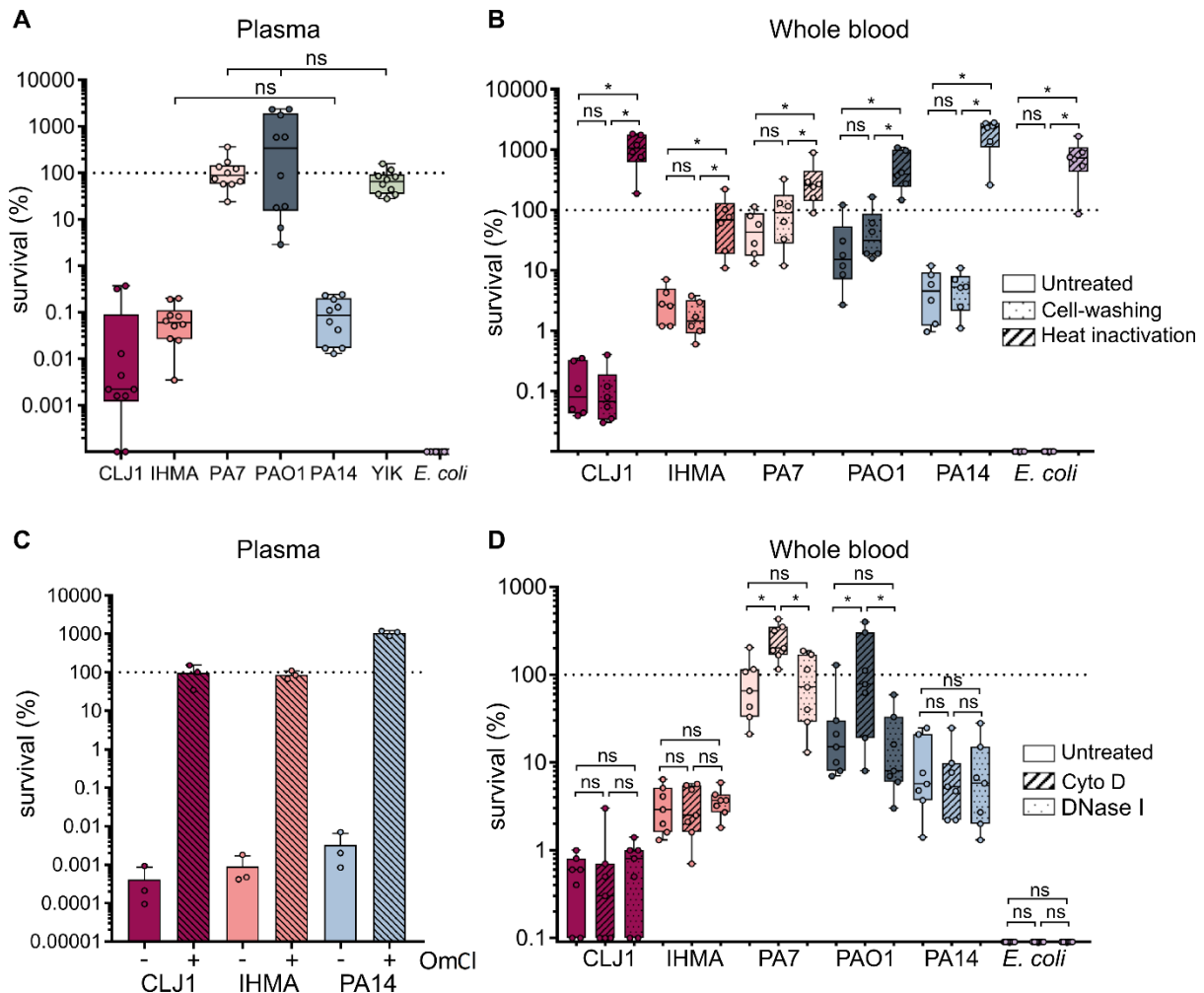


Fig2. Complement is the main immune factor involved in bacterial clearance in HWB. (A) Survival of *P. aeruginosa* strains in human plasma. Strains were incubated in plasma from different healthy donors (n=10), and survival was measured based on CFU counts. ns: non-significant. When not stated, differences in survival were significant. Kruskal-Wallis test, $p < 0.001$; Student-Newman-Keuls post-hoc test: $p < 0.05$. Note similarities in survival profiles to those shown in Figure 1. **(B)** Heat-treating plasma prevents elimination of bacteria from HWB. Bacteria were incubated for 3 h in HWB from different donors (n=6) with (hashed) and without (solid) heat-treatment to inactivate complement, or only with washed blood cells without plasma heating (dotted). The effect of this treatment on bacterial survival was assessed based on CFU counts. **(C)** Inhibition of MAC formation rescues *P. aeruginosa* strains from killing. Bacteria were incubated in a pool of human plasma for 3 h with (hashed) or without (solid) OmCI at 20 $\mu\text{g}/\text{mL}$ (IHMA87 and PA14) or 40 $\mu\text{g}/\text{mL}$ (CLJ1) and survival was measured based on CFU counts. Due to bacterial clumping, sonication was used to increase the accuracy of CFU measurement of IHMA87, as described in Methods. Data represent mean \pm SD of three independent experiments. **(D)** Phagocytes are involved in the elimination of a limited number of strains. Bacteria were incubated for 3 h in HWB from different donors (n=7) in the absence (solid) or presence of Cytochalasin D (hashed) or DNase I (dotted), to monitor the impact of these treatments on strain survival. **(B)** and **(C)**: Kruskal-Wallis test, $p < 0.05$; Student-Newman-Keuls post-hoc test: * $p < 0.05$. Dots on the x-axis correspond to no detectable colonies.

Complement evaders display persister-like features

As indicated above, only the laboratory strain *E. coli* CF7968 was entirely eliminated upon exposure to plasma. The subpopulation of *P. aeruginosa* evaders in this environment ranged from 0.1% down to 0.002% of the initial bacterial load, depending on the strain (Fig 2A). We further investigated this intriguing difference in sensitivities using the three complement-sensitive strains PA14, CLJ1, and IHMA87, by carefully examining the kinetics of bactericidal activity in plasma over a 6-h incubation (Fig 3A). A biphasic curve of bacterial killing was observed, with the majority of the sensitive population (> 99.9%) eliminated within 2 h. Following this first phase, killing slowed down, reached a plateau and left a minor subpopulation of surviving cells. However, this subpopulation failed to grow, even after 6 h. We verified that the drop off in killing rate was not due to depletion of complement activity after 2 h by retesting the used plasma. The used plasma was still sufficiently active to kill > 10⁷ *P. aeruginosa* PA14 cells during a 1-h incubation (Fig S3A). Thus, the plasma had a residual bactericidal capacity, sufficient to eliminate a population at least 4-log more numerous than the number of evaders. As further evidence that evaders are not simply a result of bacterial overload of the complement system, inoculating HWB with 10-fold fewer bacterial cells resulted in the same proportion of evaders (Fig S3B). Based on these results, evaders correspond to phenotypic variants displaying complement tolerance, and appear to be present in similar proportions to antibiotic-tolerant persisters [43,44].

To further phenotypically characterize evaders, we re-cultured the survivors recovered from a first incubation in plasma and challenged their progeny. As shown in Fig 3B, following re-culture, the bacterial population had a similar sensitivity profile to previously unchallenged cells. In some cases, the number of evaders in these repeat challenges was below the limit of detection in our experimental settings (e.g. CLJ1 after 2 h). This apparent concern is a hallmark in antibiotic persisters research [45]. Thus, neither evaders nor persisters reflect the emergence of resistant mutants, rather the evaders' phenotype, similar to persisters', is transient and reversible.

As complement evaders were detected for all sensitive *P. aeruginosa* strains, we tested whether this behavior could be extended to other Gram-negative bacteria. To that aim, we selected strains of seven Gram-negative species (Table S1) and assessed their survival in pooled plasma. Among the strains tested, *Stenotrophomonas maltophilia* was undetectable after 1 h of incubation, and *Serratia marcescens* presented what we called a tolerant phenotype in plasma, with a slow but constant elimination rate. In contrast, the five other strains tested – *Acinetobacter baumannii*, *Burkholderia multivorans*, enteroaggregative (EA) *E. coli* 17-2, *Klebsiella pneumoniae*, and *Yersinia enterocolitica* – presented a biphasic survival curve similar to the one recorded for *P. aeruginosa* (Fig 3C). For these strains, the proportion of evaders withstanding plasma-mediated killing after 3 h of incubation ranged from 1% to 0.0002% of the initial population. When individual evader colonies were re-cultured, a population as sensitive as the parental one was recovered, as seen for *P. aeruginosa*. Unexpectedly, for *K. pneumoniae*, one evader colony out of the three that were randomly selected gave rise to a resistant population (Fig 3D). Thus, plasma-resistant mutants can be selected and we do not currently know whether selection occurs over the course of the pre-challenge culture steps, or during contact with plasma.

As with *P. aeruginosa*, we verified that complement was the main driver of the elimination of these plasma-sensitive strains by using OmCI. Prevention of MAC formation abolished elimination of *A. baumannii*, *E. coli* 17-2 and *Y. enterocolitica*, whereas it only partially rescued *B. multivorans* and *K. pneumoniae*, for which the number of surviving cells increased by 190 and 40 times, respectively. However, their overall survival was still below 0.05% (Fig S4). We

conclude that depending of the strain, plasma evaders are either exclusively or only partly composed of complement-tolerant bacteria, the later observation suggesting that other fluid-phase effectors can be involved in the selection of this subpopulation.

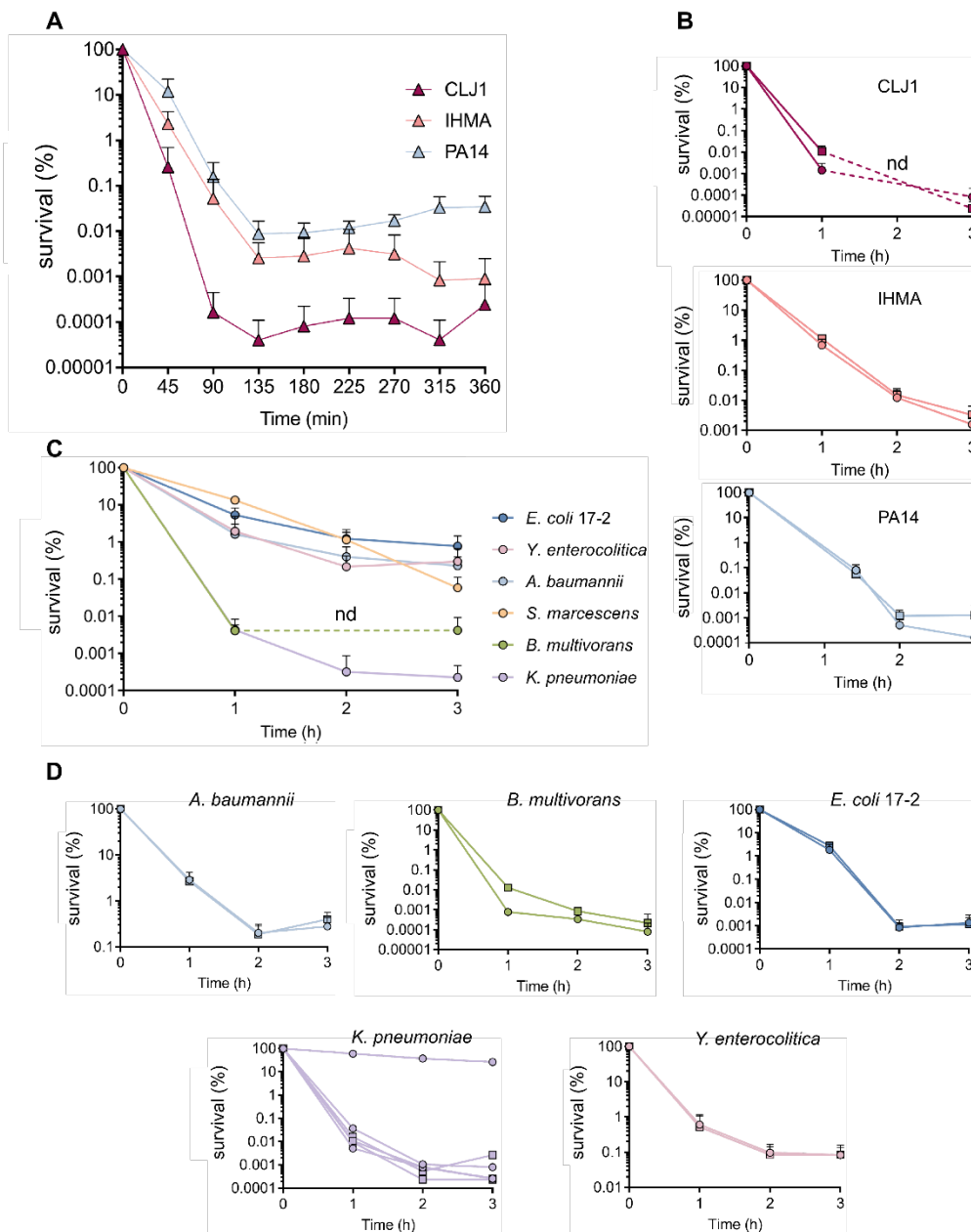


Fig3. Evaders are rare, complement-resistant, phenotypic variants. (A) Prolonged exposure of *P. aeruginosa* to plasma results in killing curves reminiscent of those that characterize persister formation. The three complement-sensitive strains PA14, CLJ1 and IHMA87 were incubated for 6 h in a pool of human plasma, and their survival was measured every 45 min to determine the kinetics of their survival. (B) The evader phenotype is reversible and not the result of fixed mutations. Following a first challenge, a single “evader” colony was re-cultured to assess the survival of its progeny (circle) in pooled plasma during 3 h. Survival was compared to that of a population that was never exposed to complement (square). (C) Evaders are a common feature among Gram-negative bacteria. *A. baumannii*, *B. multivorans*, enteroaggregative *E. coli*, *K. pneumoniae*, and *Y. enterocolitica* were incubated in pooled human plasma for 3 h, and their survival was assessed hourly. (D) The reversibility of the

tolerant phenotype was assessed in individual colonies as in **(B)**. Data represent mean \pm SD of three independent experiments **(A to D)**. Note that for *K. pneumoniae* in **(D)**, the three experimental points were not pooled as one resistant mutant was isolated, presenting a survival profile different from the two other evader clones. nd: non-detected.

Evaders are not dormant cells

To further describe the features of complement evaders, we assessed if these rare bacteria could arise in conditions known to trigger the emergence of antibiotic-tolerant persisters. Antibiotic persisters can correspond to up to 100% of cells in the stationary phase of growth [46], presumably due to growth arrest [47–49]. Thus, after verifying bacterial growth-rates and states (log versus stationary) (Fig S5), we tested whether the evaders observed in exponentially growing cultures corresponded to residual non-growing cells from the previous overnight culture, or to rare cells that had already entered the stationary state after a few hours of culture. To eliminate possible artefacts due to the growth phases [45,50], we challenged stationary-phase cells or exponentially growing cells with plasma. For the three strains *P. aeruginosa* IHMA87, *A. baumannii* and *Y. enterocolitica*, we observed that the level of evaders was higher in actively growing cultures, while for the other strains their proportions were similar between the two states (Fig 4A). Therefore, exponentially growing bacteria had a similar or higher capacity to produce evaders, suggesting that the emergence of evaders is unrelated to dormancy before complement challenge. To assess whether metabolic shut-down could increase the proportion of evaders, prior to exposure to plasma, *P. aeruginosa* IHMA87 was treated with the protonophore cyanide m-chlorophenylhydrazine (CCCP), which uncouples oxidative phosphorylation. Exposure to CCCP has been reported to increase the proportion of antibiotic persisters in *P. aeruginosa* by almost 2-log [51]. The addition of CCCP had no effect on bacterial survival in LB (Fig S6), but its use before the plasma challenge completely abolished the detection of evaders after 2 h of incubation (Fig 4B), suggesting that proton-motive force is necessary for survival. To assess more growth parameters of evaders and the possibility that they display a ‘slow growth phenotype’, we performed time-lapse microscopy of IHMA87-GFP bacteria on agarose pads after incubation in normal or heat-inactivated plasma (Fig 4C to 4F). Our results show that evaders immediately and actively grow following plasma removal and spotting on agarose pads, forming exponentially-growing microcolonies (Fig 4C). In both normal and heat-inactivated plasma-treated conditions, growth rate was low upon spotting (0.6 ± 0.3 h⁻¹) but stabilized at levels similar to batch cultures (1.2 ± 0.1 h⁻¹) after 2.5 h (Fig 4D). Division was not impaired in evaders as the mean time to reach a first division did not vary between normal (1.08 ± 0.35 h) and heat-inactivated plasma-treated cells (0.98 ± 0.34 h) (Fig 4E and 4F). Altogether, these results suggest that survival to plasma treatment is not dependent on growth rate but requires energy and active metabolism.

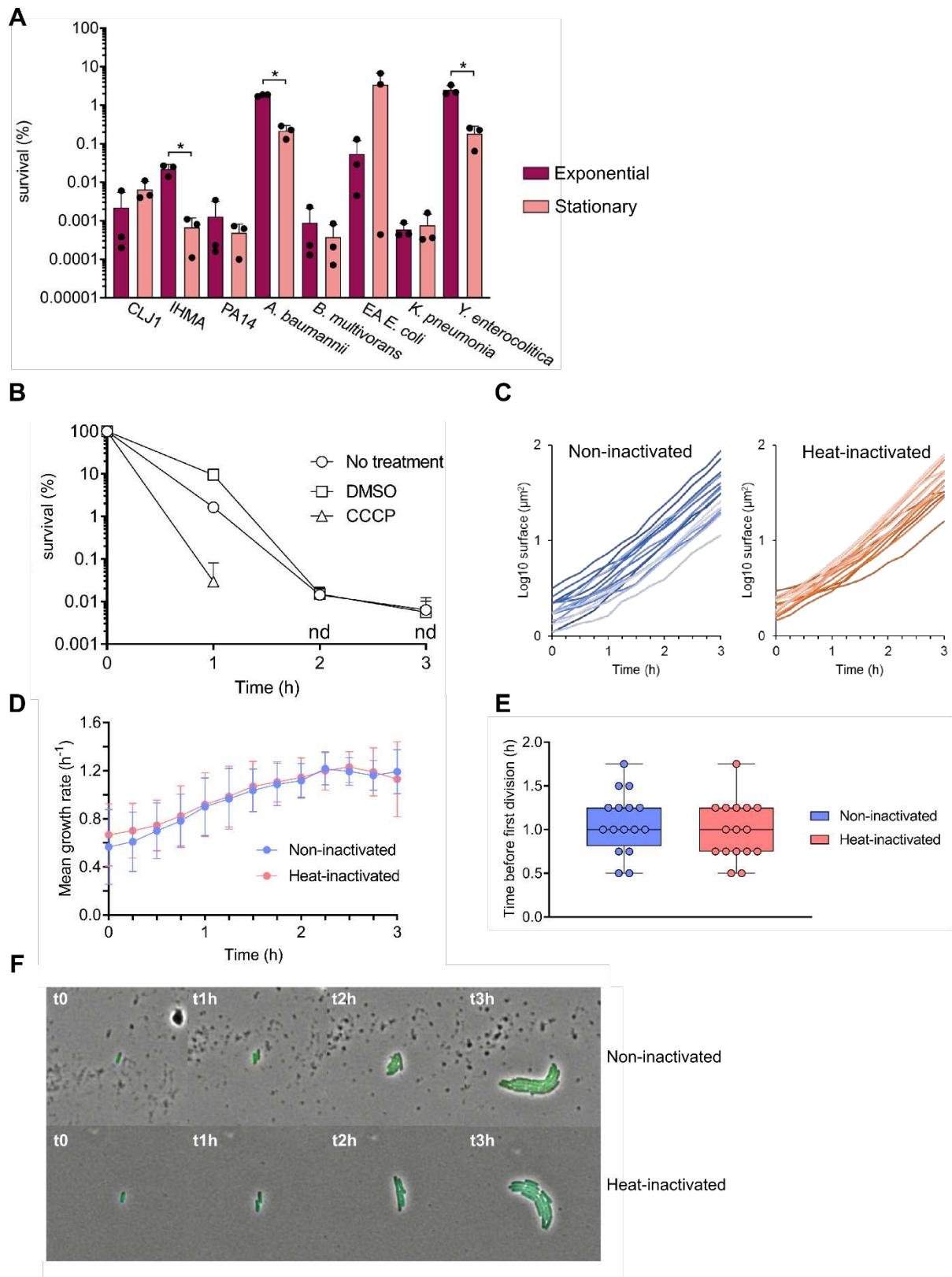


Fig4. Complement evaders differ from antibiotic persisters. (A) Cells in the stationary phase do not form more evaders than exponentially growing bacteria. Bacteria from stationary phase and exponentially growing cultures from every evader-forming strain were challenged in a pool of plasma for 3 h to compare their ability to form evaders. Student's t-test: * $p < 0.01$, performed on log-transformed values. (B) Formation of evaders requires active metabolism. Survival kinetics of IHMA87 in a pool of plasma, following 1-h treatment with the protonophore CCCP

in LB. As controls, bacteria in LB were either not treated or incubated 1 h with 0.5% DMSO prior the challenge. nd: non-detected. **(C) to (E)** Following stress removal, evaders show no growth defect. After 3 h incubation of IHMA87-GFP in pooled plasma following heat-inactivation or not, surviving cells were recovered and spotted on a 2% agarose pad containing LB, allowing the bacteria to regrow. Using time-lapse microscopy, individual microcolony surface **(C)**, mean growth rate **(D)** and elapsed time before the first division **(E)** were determined. From two independent experiments, 16 evader cells were analyzed and compared with 16 non-evader cells from the control condition (heat-inactivated plasma). **(F)** Time-lapse microscopy of two representative microcolonies from each condition. Data represent mean \pm SD of three independent experiments **(A and B)**.

BSI isolates show full range of plasma resistance and presence of evaders

As we had demonstrated that the central driver of bacterial clearance from blood was the complement system, mainly through its direct lytic activity, we next investigated survival in plasma of a cohort of twelve clinical strains isolated from patients with BSI (Table S1 and S2) to determine their capacity to form evaders. Like the data obtained with the initial six selected strains, BSI isolates displayed differences in survival rates in plasma, of up to five orders of magnitude. Four strains (PaG2, PaG5, PaG6, and PaG10) were tolerant to killing, displaying > 50% survival, with some even able to multiply in these conditions (PaG2, PaG6 and PaG10) (Fig 5A). In contrast, for other strains (PaG8, PaG9, PaG14) just < 0.02% of the initial population survived (Fig 5A). The limited number of strains and the high diversity of serotypes identified (Table S1) would make any attempt to correlate bacterial survival in plasma with strain serotype too speculative. Even though some strains were highly sensitive to plasma, none was fully eliminated. To verify that these surviving cells corresponded to evaders, we also assessed their survival kinetics in plasma (Fig. 5B). The three isolates PaG3, 9, and 17 presented a tolerant phenotype, with a constant rate of elimination, never reaching a plateau even after 4 h incubation. In contrast, a biphasic killing curve was recorded for the five other isolates, the kinetics of the curve varied from strain to strain, sometimes reaching a plateau after just 1 h, whereas for others the death rate started to slow from the 3-h time point. As indicated above, in some cases, surviving cells were scarce and below the limit of detection (e.g. PaG8 at 2 and 3 h). Thus, most clinical isolates form evaders that can withstand the bactericidal property of plasma, suggesting that this phenomenon could be exploited by *P. aeruginosa* to persist within the bloodstream in clinical settings.

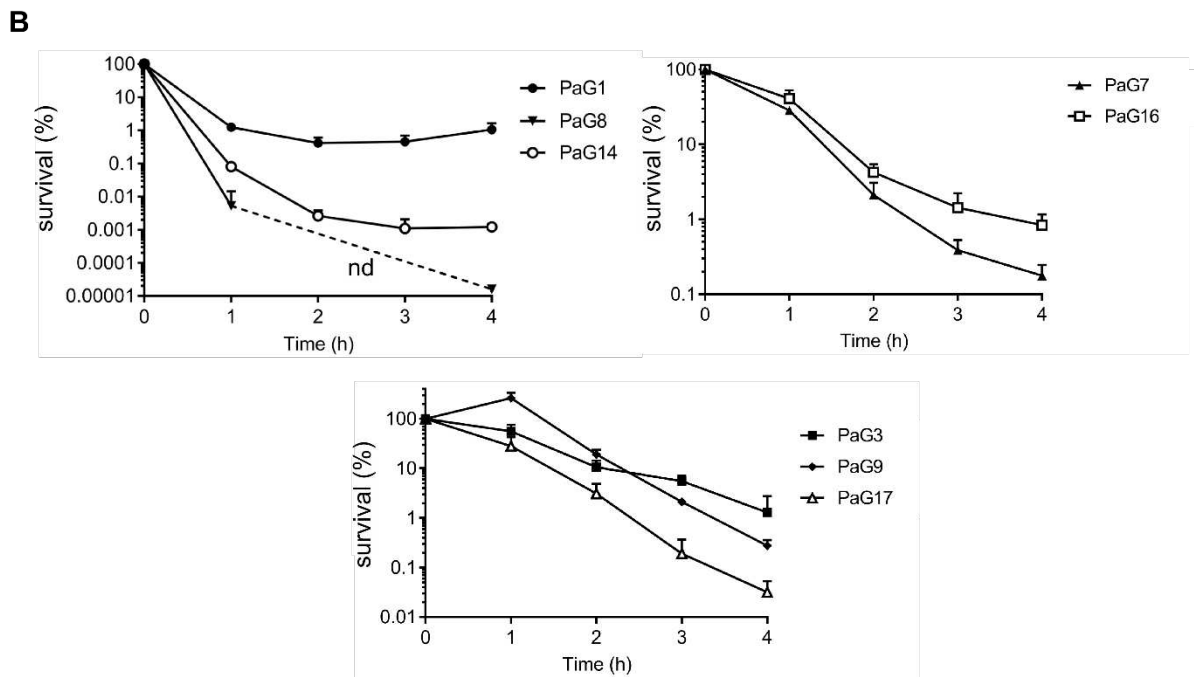
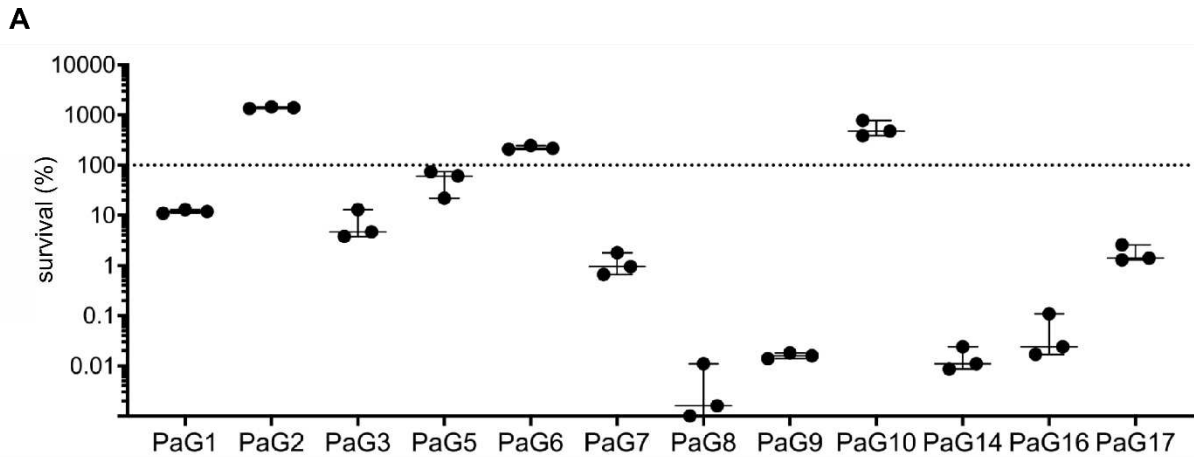


Fig5. BSI isolates avoid elimination mainly through complement evaders. (A) Complement-resistance is not common to all BSI isolates. Twelve *P. aeruginosa* isolates from bloodstream infections were incubated in pooled plasma for 3 h, and their survival was assessed at the end of the incubation. Dots on the x-axis correspond to no detectable colonies. **(B)** Most complement-sensitive isolates form evaders. Kinetics of survival in pooled plasma for 3 h of eight complement-sensitive clinical isolates. Three different panels are used for clarity and to allow better visualization of the inflection points. nd: non-detected. Data represent mean \pm SD of three independent experiments (**A** and **B**).

Discussion

Bacteremia caused by the multi-drug resistant opportunistic pathogen *P. aeruginosa* presents a particular threat to hospitalized patients. To gain more knowledge on host and pathogen strategies associated with BSI, we undertook an extensive analysis of a cohort of *P. aeruginosa* strains in an *ex vivo* HWB model of infection. Although many previous studies have addressed bacterial transmigration across epithelial and endothelial barriers, the interplay between the immune system and bacterial survival in HWB has been less extensively documented. We found that the nature of major *P. aeruginosa* toxins ExoS, ExoU or ExlA was unrelated to a strain's capacity to survive in HWB, even though these toxins play important roles in breaching epithelial and endothelial barriers [13,27,31,52,53]. The levels of bacterial survival measured in HWB were highly variable and directly related to the action of the complement system, as previously reported for carbapenem-resistant epidemic clones of *Klebsiella pneumoniae* [54]. NETs did not contribute significantly to bacterial killing. In some cases, PMNs contributed to bacterial elimination through complement-dependent opsonophagocytosis, but this process was less efficient than direct MAC-induced lysis. In line with this observation, Thanabalasuriar and colleagues [55] observed that neutrophils had a limited capacity to phagocytose *P. aeruginosa* in the mouse lung vasculature, in contrast to PMNs recruited to the organ at the site of infection [56]. Complement resistance is the main driver of survival in the bactericidal environment that human blood represents. Nevertheless, among the tested isolates, we observed that to fully resist the immune system, the strains had to display both complement resistance and cytotoxicity toward professional phagocytes, as documented for the highly virulent YIK strain recently isolated from a 49-year-old individual with no known immunodeficiency [29]. Whether these characteristics apply to other clinical isolates able to survive in the blood should be investigated on bigger bacterial cohorts.

A plethora of previously identified bacterial factors could interfere with the complement system in the HWB model. Thus, bacteria could alter C3b binding [19,36,55,57–60], recruit negative complement regulators [18,20,61,62], break down complement proteins [15,63,64], or stabilize outer membrane integrity [65,66]. Consequently, bacterial resistance to complement activity appears to be multifactorial and strain-dependent, and should thus be investigated in a more systematic and uniform way to obtain a better overall picture of the complex interactions involved. Indeed, a very recent Tn-Seq approach performed in parallel on four *Klebsiella pneumoniae* strains revealed a few general, but mainly strain-dependent, factors contributing to complement-resistance [67].

The most prominent result from our study is the evidence that strains highly sensitive to plasma can nevertheless escape complement's bactericidal activity by forming phenotypic variants, or evaders. In some instances, complement evaders represent < 0.01% of the initial population, with survival kinetics reaching a plateau reminiscent of bacterial persisters following antibiotic challenges [45]. As with antibiotic persisters [45], we found that complement evaders lose their complement-tolerant phenotype upon elimination of the stress. However, complement evaders do not form under conditions previously described as triggering the appearance of antibiotic persisters. Indeed, persisters are more numerous in stationary-phase cultures [49], whereas the fraction of evaders is similar or even higher in exponentially growing cells. CCCP treatment, which decreases ATP production by membrane-bound ATPases as a result of inhibition of proton motive force was previously shown to increase the number of persister cells by 2-log upon challenge with ciprofloxacin [51]. Here, metabolic shut-down with CCCP prior to the plasma challenge abolished the emergence of evaders. Finally, we observed no growth defect/delay of these rare cells following removal of the stress. These results evidence that complement evaders are not in a dormant state, and that they emerge through an energy-

dependent process. Based on these results, we propose that complement evaders share only some characteristics with antibiotic persisters to withstand a stress [43,68–71], however the mechanisms leading to the development of these traits remain a subject of debate [72], and might greatly depend on the nature of the challenge faced by the bacteria.

Interestingly, following serum challenge of uropathogenic *E. coli*, Putrinš and colleagues [73] reported the emergence of complement-resistant stationary-phase persisters. This group also identified a subpopulation of non-quiescent cells undergoing rapid division in serum, which could withstand complement-mediated lysis but was sensitive to antibiotics. This population may be the same as the evaders described here. However, Putrinš and colleagues only observed these cells from stationary-phase cultures, as exponentially growing bacteria appeared to be serum-resistant in their experiments. These results might suggest that, in addition to complement evaders, bacteria may have developed a number of ways to transiently hide from the immune system when present in the blood. Whether complement evaders are also antibiotic-tolerant requires further investigation.

Host immunity can amplify a pathogen's phenotypic heterogeneity, promoting the formation of antibiotic persisters both *in vivo* and *in vitro*. For example, upon lung infection with *M. tuberculosis*, cell-to-cell variations in ribosomal RNA transcription patterns increased markedly compared to growth-permissive *in vitro* conditions [74]. Bacterial uptake by macrophages was also recently shown to induce persistence in both *Salmonella enterica* and *S. aureus*, in response to the stress conditions encountered during vacuolar internalization [75–77]. Exposure to human serum has also been linked to an increased frequency of antibiotic persisters in *Vibrio vulnificus* [78]. The process involved in the emergence of this population is at least partly mediated by complement activity, as a lower proportion of surviving cells was detected following exposure to heat-inactivated serum [78]. Our results show that human plasma can trigger phenotypic diversity in addition to antibiotic persistence, although we still lack information on whether the evader phenotype emerges spontaneously (are these cells already present in the population prior to the stress?) or in response to a trigger (do they only appear upon contact with complement?) [50]. Ongoing experiments should reveal whether evaders activate less the complement system, or whether they harbor an increased tolerance to MAC insertion at their surface. As these bacterial cells also persist in blood, we hypothesize that lower amount of C3b deposits at their surface; otherwise, they should be efficiently phagocytosed by circulating neutrophils. This minor population of evaders has been ignored so far as it can represent less than 0.01% of the initial population, and because bacterial survival in serum is often monitored at a single time point (usually after a 1-h challenge), rather than examining the kinetics of survival over a long enough period to reach a plateau.

Although BSIs were historically considered “dead-ends” for infectious agents, Bachta and colleagues [23] recently reported that once in the blood, a subpopulation of *P. aeruginosa* migrates to the gallbladder in mice, where it replicates and can exit the organism through the intestinal tract, causing contamination of cage-mates. In our experiments, not all BSI isolates were complement-tolerant, but most of the sensitive strains could form evaders, making the bacteria potentially transmittable.

Numerous bacterial pathogens affecting humans may form an evader population, which could represent a reservoir of complement-tolerant cells capable of disseminating and spreading throughout the organism. By elucidating the molecular mechanisms through which complement evaders emerge, notably by performing transcriptomic/proteomic profiling of these populations, we hope to identify ways to diminish the risks of bacteremia caused by various bacterial pathogens.

Methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table S1. Bacteria were grown in liquid Lysogeny Broth (LB) prepared according to Miller's formulation (0.5% yeast extract, 1% tryptone, 10% NaCl) for >16 h with agitation (300 rpm) at 37 °C, except for *Y. enterocolitica*, which was cultured at 28 °C. If not otherwise specified, the overnight culture was diluted approx. 40 times in LB to have an initial OD_{600nm} ~ 1 and placed at 300 rpm at 37°C until the OD_{600nm} reached ~ 1.

Whole blood and plasma killing assays

Heparinized HWB from healthy donors was provided by the French National blood service (EFS, Grenoble, France) and was used within 3 h of collection. Bacteria resuspended in RPMI (Thermo Fisher Scientific, Illkirch, France) were incubated in HWB (90% final blood concentration) at a theoretical multiplicity of infection (MOI) of 5 per phagocyte (monocytes and granulocytes), which corresponded to a final bacterial concentration of 2.25×10^7 mL⁻¹. The precise value was verified for each experiment by plating the bacteria on LB agar plates at t_0 and counting colony-forming units (CFU) after ~ 15 h incubation at 37 °C. Tubes were incubated for 3 h on a rotating wheel at 37 °C in a 5% CO₂ atmosphere. Following incubation, bacterial survival was determined following serial dilutions in H₂O by colony counting on LB or selective PIA (Pseudomonas Isolation Agar) medium. Bacterial survival was expressed as a percentage (%) of survivors calculated from the CFU number after 3 h (t_{3h}) incubation relative to the CFU measured in the initial inoculum (t_0). To inhibit the potential bactericidal effect of phagocytosis and Neutrophil Extracellular Traps (NETs), Cytochalasin D (10 μM) and DNase I (200 U/mL) (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were applied to the HWB for 30 min at 37 °C prior to the addition of bacteria. In the non-treated condition, RPMI medium without inhibitors was added to the blood.

To inactivate complement, HWB was centrifuged for 5 min at 400 g to isolate plasma, which was subsequently heat-inactivated for 30 min at 56 °C. Meanwhile, cells were washed twice with RPMI and pelleted by centrifugation for 5 min at 400 g. Complement-inactivated whole blood was reconstituted by mixing the heat-inactivated plasma with the washed cells. A control condition was also prepared, combining washed cells with untreated plasma.

For plasma killing assays, heparinized HWB was centrifuged for 10 min at 1000 g. The supernatant was recovered and filtered through a 0.2-μm membrane prior to storage at -80 °C until needed. Pools of plasma used in experiments were from ten individual donors. After thawing, plasma was systematically filtered once again through a 0.2-μm membrane. Bacteria in PBS supplemented with calcium and magnesium (Thermo Fisher Scientific, Illkirch, France) were incubated in plasma (90% final plasma concentration) at the same concentration as that used for whole blood assays (2.25×10^7 CFU/mL). At various time points (see figures), survival was determined by counting colonies on LB or PIA, following serial dilutions in PBS supplemented with calcium and magnesium. As for the HWB assays, the CFU count for the starting inoculum was taken as reference (100% survival) to quantify bacterial killing.

To prevent MAC formation and subsequent bacterial killing by complement, plasma was incubated 5 min at 37°C in presence of complement-inhibitor OmCI (kindly provided by Suzan H.M. Rooijackers and Maartje Ruyken, University Medical Center Utrecht, The Netherlands) prior to the addition of bacteria. Depending on the strain's sensitivity in plasma, OmCI was used either at 20 μg/mL (*P. aeruginosa* strains IHMA87 and PA14, *A. baumannii* and *Y.*

enterocolitica) or 40 µg/mL (*P. aeruginosa* CLJ1, *B. multivorans*, EA *E. coli* 17-2 and *K. pneumoniae*). Due to aggregation of IHMA87 and to avoid artifacts of CFU counting, additional sonication step was added. Following 3h incubation in plasma with or without OmCI, 100 µL samples were transferred in 200 µL PCR tubes and placed in ultrasonic bath of the sonicator Q700 (Qsonica, Newtown, USA). Following the manufacturer instructions, tubes were placed at 2 mm from the probe, to increase the efficiency and reproducibility of the treatment. Sonication was performed at 6°C for 45 sec at 10% intensity (5 sec pulse and 5 sec rest).

Cyanide m-chlorophenylhydrazone treatment before plasma challenge

CCCP (Sigma-Aldrich, Saint-Quentin-Fallavier, France) solubilized in dimethyl sulfoxide (DMSO) was added to the LB culture once an $OD_{600nm} \sim 1$ was reached. The final CCCP and DMSO concentrations were 200 µg/mL and 0.5%, respectively. Following 1 h incubation at 37 °C with agitation (300 rpm), bacteria were recovered by centrifugation for 5 min at 5,000 g. The bacterial pellet was resuspended in PBS supplemented with calcium and magnesium. As controls, bacteria were either not treated or exposed 1h in LB with 0.5% DMSO.

Cytokine quantification in whole blood

Cytokine concentrations in whole blood were assessed by flow cytometry. Briefly, bacteria suspended in RPMI were added to HWB (2.25×10^7 CFU/mL) and incubated for 3 or 6 h. Plasma was recovered following a 5-min centrifugation step at 400 g and stored at -80 °C until required. Analytes were quantified using the LEGENDplex Human Inflammation Panel 1 (Biolegend, San Diego, USA) according to the supplier's instructions, and samples were analyzed on a FACSCalibur (Becton Dickinson, Pont de Claix, France). For the non-infected control (NI), RPMI was added to the blood.

Neutrophil and peripheral mononucleated cells isolation from blood

Circulating leukocytes were purified from heparinized blood of healthy donors as previously described [79]. Plasma was recovered following a 5 min centrifugation (room temperature, 800 g), and cells were resuspended in PBS with Trisodium citrate 1% (w/v). This suspension was carefully layered on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) with a density of 1.077g/cm³. After a 20 min centrifugation (20°C, 400 g), the PBMC ring was recovered and subsequently washed in RPMI 1640 medium (Thermo Fisher Scientific, Bourgoin-Jallieu, France). The cell pellet (red blood cells and granulocytes) was resuspended in cold lysis buffer (0.155M NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) and incubated 15 min to allow complete lysis of erythrocytes. Purified granulocytes were subsequently washed in RPMI 1640 medium. Purity (>95%) of isolated leukocytes was assessed by flow cytometry on a FACSCalibur based on FSC/SSC parameters. Viability of purified leukocytes was determined by Trypan Blue exclusion and was >95%.

LDH release by purified leukocytes

PMNs or PBMCs (4×10^6 cells) were incubated with each strain of interest at a MOI of 5 for 3 h in RPMI supplemented with 10% heat-inactivated fetal calf serum on a rotating wheel at 37 °C in a 5% CO₂ atmosphere. Supernatants were then recovered to quantify LDH release using the cytotoxicity detection kit (Roche, Meylan, France). As a positive control (100% cytotoxicity), cells were incubated with 1% Triton X-100, while the basal level of LDH release was determined by incubating cells without bacteria.

Time-lapse microscopy to assess the regrowth of complement-resilient bacteria

After incubation in normal plasma (or in heat-inactivated plasma for the control condition), bacteria were collected by centrifugation for 5 min at 5,000 g, washed and resuspended in PBS and then spotted on a 2% agarose pad containing LB. After complete absorption of the liquid into the pad, the preparation was sealed under a 0.17-mm glass coverslip using an adhesive plastic frame (Gene Frame 125 μ L, Thermo Fisher Scientific, Illkirch, France). Time-lapse microscopy was performed at 37 °C using a 100x oil immersion objective on an inverted microscope (Axio Observer Z1, Zeiss, Germany). Phase-contrast and fluorescent images were recorded every 15 min with a Hamamatsu ORCA-Flash 4.0 digital camera and a Zeiss 38HE filterset. In each experiment performed (two for normal and two for heat-inactivated plasma), eight isolated microcolonies were segmented using MicrobeJ (27572972) to determine their surface, growth rate and time of first division.

Serotyping of BSI isolates

The twelve clinical isolates were serotyped using 16 monovalent antisera directed against *P. aeruginosa* LPS (Bio-Rad, Marnes-la-Coquettes, France), according to the manufacturer's instructions.

Statistical analysis

Statistical tests were performed using SigmaPlot software. To analyze multiple comparisons, a one-way ANOVA or Kruskal-Wallis test were applied, depending on the normality of the data. A Student-Newman-Keuls pairwise comparison was then performed. Mann-Whitney U test or Student's t-test were used to compare two groups, depending of the normality of the data. Where indicated, values were log-transformed to convert initially non-normally-distributed data into a normally-distributed dataset. GraphPad Prism was used to create graphs.

Ethics statement

Medical data from patients infected with *P. aeruginosa* strains were extracted from medical records. No nominative or sensitive personal data were recorded, and the study only involved the reuse of already available data. This study falls within the scope of the French Reference Methodology MR-004 for studies not involving human subjects.

Data Availability Statement

All relevant information are provided in the article and the supporting information files.

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Conflict of interest

All authors declare no competing interests.

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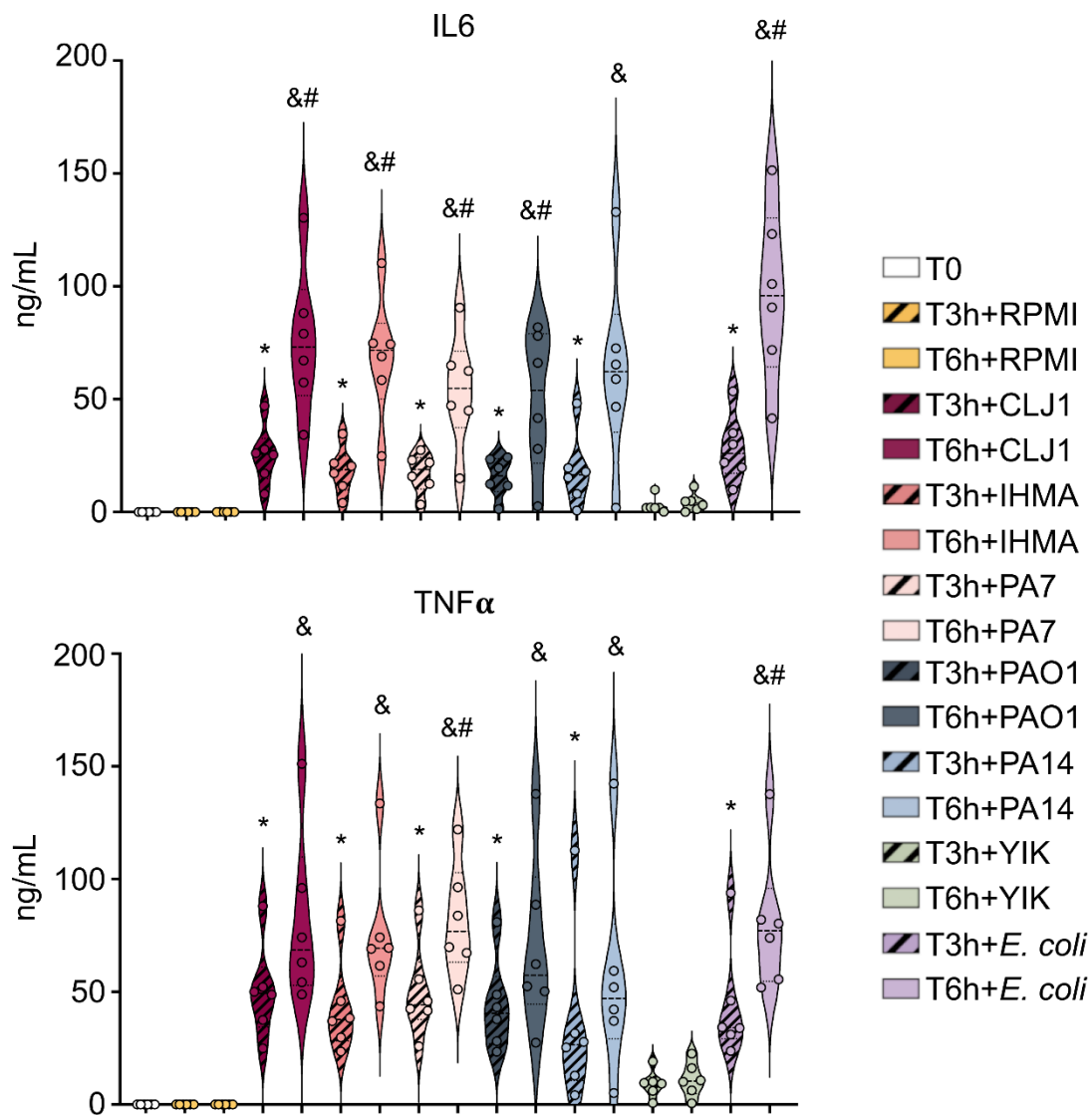
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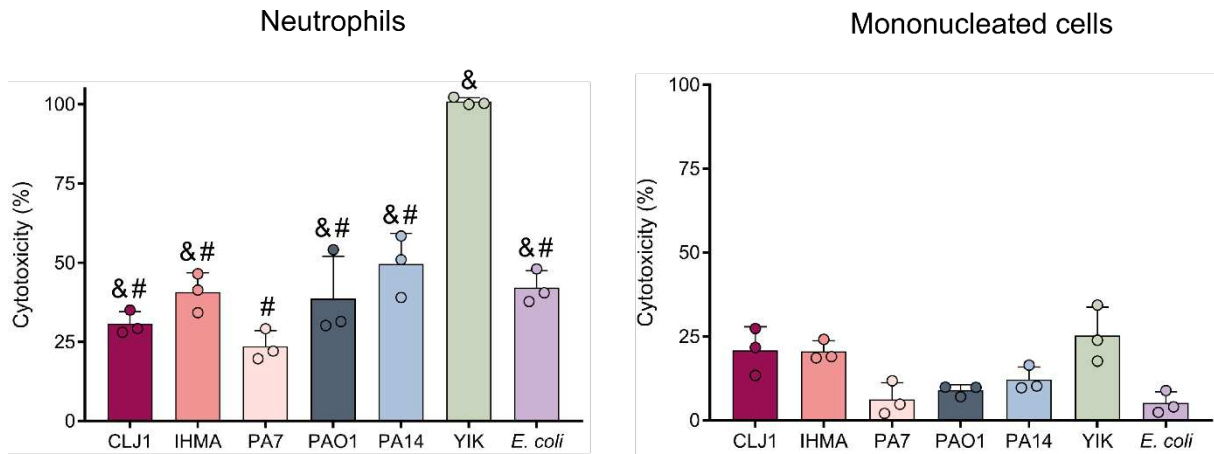
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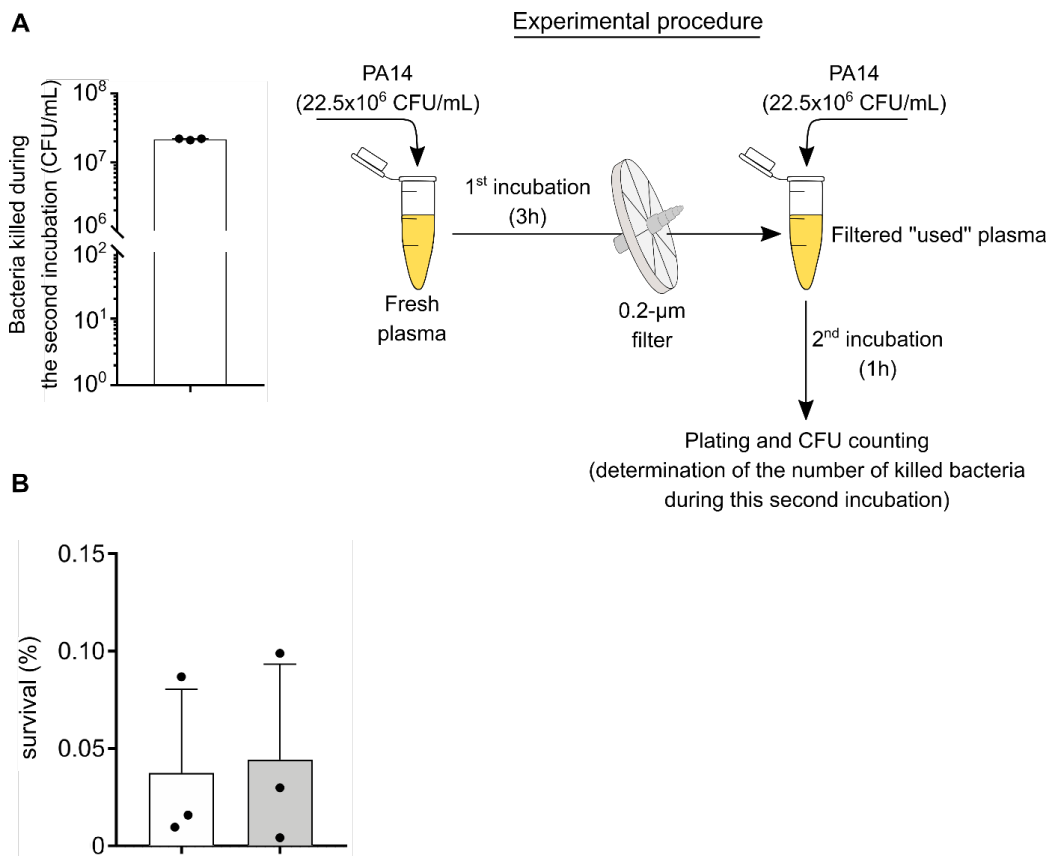
Supporting Information



FigS1. All tested strains except YIK have a similar capacity to induce cytokine release. Strains, as indicated, were incubated in HWB (n=6, from different donors) for the time points indicated, and cytokines were quantified by a multiplex analysis. (*): different from T3h+YIK. (&): different from T6h+YIK. (#): different from the cognate T3h. Mann-Whitney test: # p <0.05. Kruskal-Wallis test, p <0.05; Student-Newman-Keuls post-hoc test: * and &, p <0.05.



FigS2. All strains, except YIK, display similar levels of cytotoxicity toward circulating leukocytes. Cytotoxicity of indicated strains on purified neutrophils or mononucleated cells was analyzed by LDH release (n=3, from different donors). Following a 3-h incubation of leukocytes with bacteria at a MOI of 5, LDH released by dead cells was quantified from the supernatants. The LDH release obtained following treatment with 1% Triton X-100 correspond to 100% cytotoxicity. (&): different from PA7. (#): different from YIK. Kruskal-Wallis test, $p < 0.05$; Student-Newman-Keuls post-hoc test: & and #, $p < 0.05$. Note that none of the strains tested induced a significantly different killing of mononucleated cells.



FigS3. The presence of evaders is not due to a limited complement activity. (A) Following a first challenge, the plasma is still bactericidal. Schematic view of the experimental procedure: PA14 (22.5x10⁶ CFU/mL) was incubated in a pool of plasma for 3 h. Following the first

challenge, the plasma was sterilized by passing through a 0.2- μ m filter, and re-challenged with the same number of PA14 cells for 1 h at 37 °C. More than 20×10^6 bacteria/mL were eliminated during this second incubation, showing that the plasma is still bactericidal after 3 h of contact with the bacteria. **(B)** Reducing the bacterial load does not affect the proportion of surviving cells. PA14 was incubated in a pool of plasma at concentrations of 22.5×10^6 (white) or 2.25×10^6 (grey) CFU/mL, and survival was assessed following incubation for 3 h. The proportions of evaders were unchanged regardless of the initial bacterial load. Data represent mean \pm SD of three independent experiments **(A and B)**.

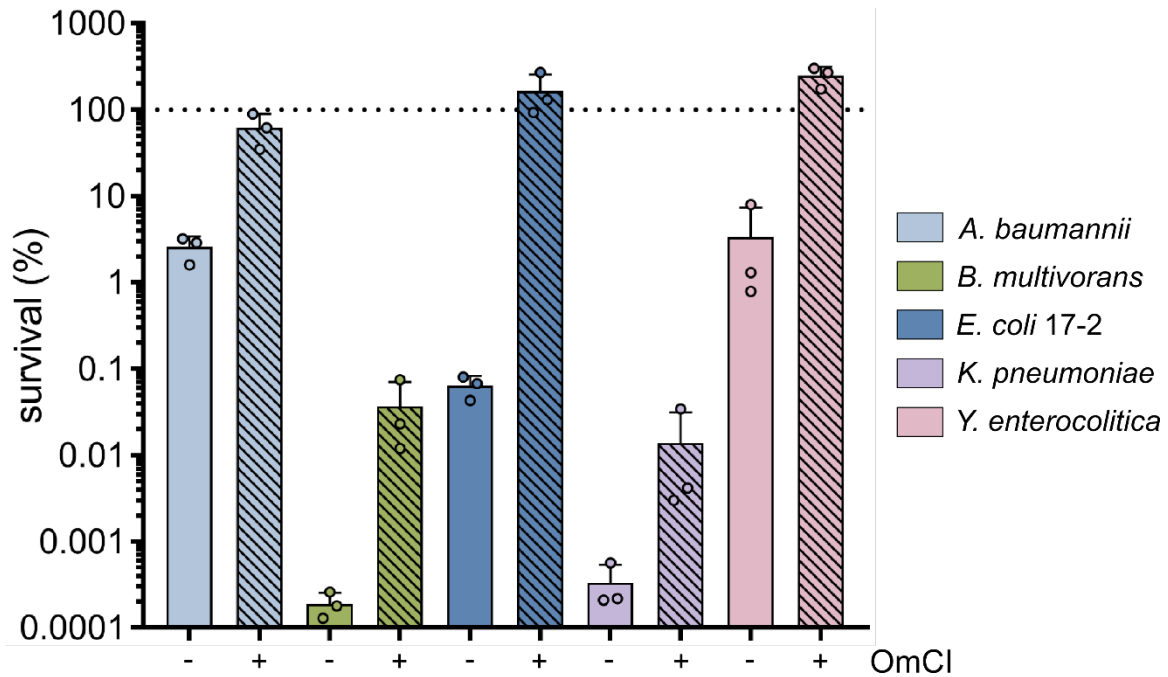
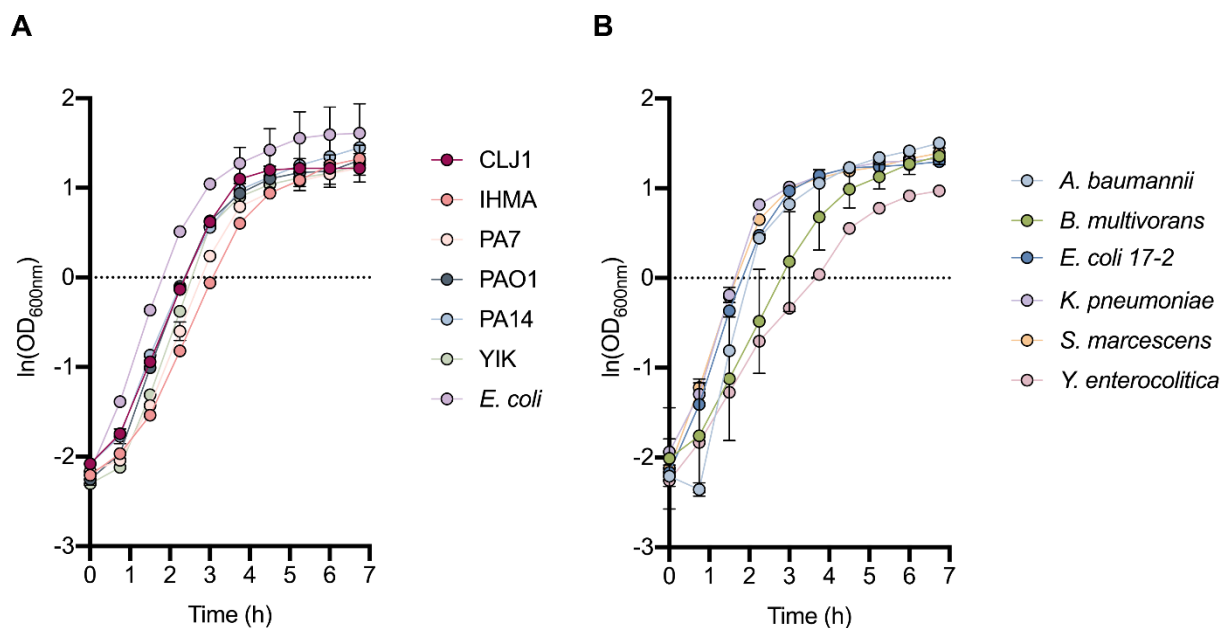
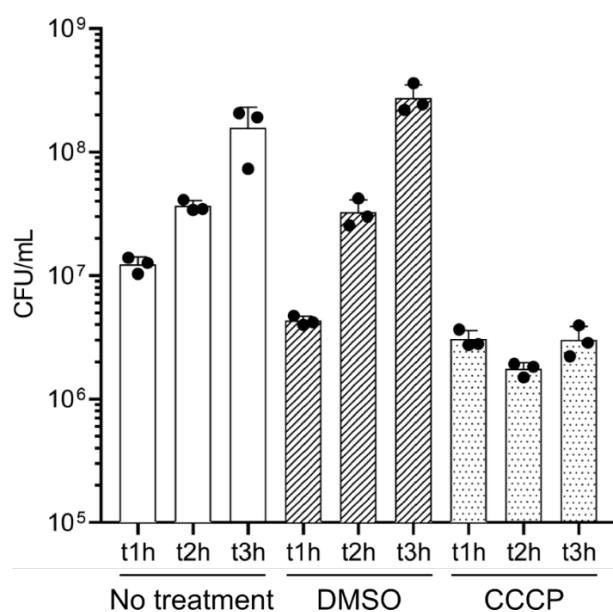


Fig S4. Plasma evaders are either solely or partly composed of complement-tolerant cells. Depending on the species, additional fluid-phase bactericidal effectors other than complement are likely involved in the selection of evaders. Bacteria were incubated in a pool of human plasma for 3 h with (hashed) or without (solid) OmCI at 20 μ g/mL (*A. baumannii* and *Y. enterocolitica*) or 40 μ g/mL (*B. multivorans*, *E. coli* 17-2 and *Y. enterocolitica*) and survival was measured based on CFU counts. Data represent mean \pm SD of three independent experiments.



FigS5. Growth curves of bacteria used in this study. Growth kinetics in LB of the six *P. aeruginosa* strains CLJ1, IHMA, PA7, PAO1, PA14, YIK, *E. coli* CF7968 (A), and the other Gram-negative species used in this study (B). Data represent mean \pm SD of two independent cultures (A and B).



FigS6. CCCP treatment efficiently shuts down bacterial metabolism but is non-toxic for the bacterial cells. Exponentially growing IHMA87 cultures in LB were either not treated (solid), treated 1 h with CCCP at 200 $\mu\text{g}/\text{mL}$ (dotted) or exposed to 0.5% DMSO for 1 h (hashed). Cells were subsequently recovered and incubated in LB alone. Evolution of the CFU number was monitored by plating after 1 h, 2 h and 3 h. Bacterial survival is unaffected following CCCP treatment, however treated cells are still non-growing even 3 h after removing the stress. At 0.5%, DMSO does not affect bacterial growth. Data represent mean \pm SD of three independent experiments.

TABLE S1. Bacterial strains used in this work

Strains	Characteristics/Source	Reference/Origin
<i>P. aeruginosa</i>		
PAO1	TTSS ⁻ , ExoS ⁻ , O5, Wound	Holloway 1955, lab collection
PA14	TTSS ⁻ , ExoU ⁻ , O10, Burn	Rahme <i>et al.</i> 1995, Lory lab
PA7	ExlA ⁺ , O12, Burn (taxonomic outlier)	Roy <i>et al.</i> 2010
CLJ1	ExlA ⁺ , O12, Hemorrhagic pneumonia	Elsen <i>et al.</i> 2014
IHMA879472 (IHMA87)	ExlA ⁺ , O11+O12, Urinary tract	Kos <i>et al.</i> 2015, IHMA*
YIK	TTSS ⁻ , ExoU ⁻ , O8, Bacteremia	Ellabadi <i>et al.</i> 2019
IHMA87-GFP	IHMA87 with constitutive expression of GFP, carrying miniCTX-PX2-GFP	This study
<i>P. aeruginosa</i> BSI isolates		
PaG1	O8, Infected organ	This study/Grenoble Hospital
PaG2	O5, “	“
PaG3	O6, Contaminated catheter	“
PaG5	O4, “	“
PaG6	O6, Infected organ	“
PaG7	O1, Contaminated catheter	“
PaG8	O4+O10, Infected organ	“
PaG9	O10, Contaminated catheter	“
PaG10	O6, “	“
PaG14	O4, Infected organ	“
PaG16	O2, Contaminated catheter	“
PaG17	O3, Infected organ	“
Other Gram-negative species		
<i>Acinetobacter baumannii</i> ATCC23220	-	Thornley 1967, lab collection
<i>Burkholderia multivorans</i> ATCC17616	-	Stanier <i>et al.</i> 1966, lab collection
<i>Escherichia coli</i> CF7968	MG1655 corrected to <i>rph</i> ⁻ and deleted for <i>lacIZ</i>	Hirsh, <i>et al.</i> 2002, D. Schneider lab
Enteroaggregative <i>Escherichia coli</i> 17-2	-	Aschtgen <i>et al.</i> 2008, E. Cascales lab
<i>Klebsiella pneumoniae</i> NCTC9633	-	Sneath <i>et al.</i> 1980, lab collection
<i>Serratia marcescens</i> Db10	-	Flyg <i>et al.</i> 1980, S. Coulthurst lab
<i>Stenotrophomonas maltophilia</i> ATCC13637	-	Hugh and Ryschenkow, 1961, lab collection
<i>Yersinia enterocolitica</i> MRS40	-	Sarker <i>et al.</i> 1998; G. Cornelis lab

*International Health Management Association (IHMA; USA)

TABLE S2. Patient demographics from which clinical BSI strains were isolated

Patient	Age	Sex	Medical unit	Comorbidities	Immuno-suppression	Sepsis	Septic shock	Source of infection	Day10 Mortality	Origin of the strain	History of <i>P. aeruginosa</i> infection
PaG1	76	H	Emergency	Malignancy	Yes	Yes	Yes	Urinary	Yes	Blood culture	Yes
PaG2	53	H	ICU	Chronic heart and liver disease, diabetes, high blood pressure	No	Yes	Yes	Ventilator-associated pneumonia	Yes	Blood culture	No
PaG3	54	F	Surgery	Malignancy	Yes	Yes	No	Hospital acquired pneumonia	No	Blood culture	No
PaG5	73	F	ICU	Chronic heart and liver disease, diabetes, high blood pressure	No	Yes	No	Central venous catheter	No	Blood culture	Yes
PaG6	81	H	Vascular medicine	Chronic heart and kidney disease, high blood pressure	No	Yes	No	Urinary	No	Blood culture	No
PaG7	43	H	Hematology	Malignancy, bone marrow transplantation	Yes	Yes	Yes	Central venous catheter or external otitis	No	Blood culture	No
PaG8	62	H	ICU	Chronic heart, kidney and liver disease ; liver transplantation	Yes	Yes	No	Peritonitis	Yes	Blood culture	Yes
PaG9	62	F	Infectious disease	Malignancy, bone marrow transplantation	Yes	Yes	No	Central venous catheter	No	Blood culture	No
PaG10	37	H	Hematology	Malignancy	Yes	Yes	No	Central venous catheter	No	Blood culture	No
PaG14	77	H	Emergency	Diabetes	No	Yes	No	Urinary	No	Blood culture	Yes
PaG16	41	H	ICU	Malignancy	Yes	Yes	No	Arterial catheter	Yes	Blood culture	No
PaG17	76	H	Vascular medicine	Diabetes	No	Yes	No	Unknown	Yes	Blood culture	No

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2) Additional Results

a) Optimization step to assess bacterial survival in human whole blood

The initial step of my project was to assess the survival of six *P. aeruginosa* and one *E. coli* strains in human whole blood. Measuring bacterial survival in a particular condition is not difficult and is usually done using a simple colony forming unit (CFU) measurement, but we wondered what was the most correct way to assess a survival rate, with the two following possibilities:

- Percentage survival compare to the initial inoculum
- Percentage survival compare to an incubation of the same length in condition with similar nutritious characteristics but without antimicrobial properties

We reasoned that the second measurement could take into account the loss of bacteria due to mechanisms unrelated to the microbicidal action of the complement/phagocytes from the blood (e.g binding of the bacteria to the tube, reduced viability because of the nutritious characteristics of the plasma), likely allowing to determine the strict effect of the immune system on the strains' survival. Because an extracellular bacterium evolving in the circulation is surrounded by plasma, we decided to take as a reference for "100% survival", the number of bacteria recovered following incubation in heat-inactivated plasma (HIP), in which the activity of the complement system is eliminated thanks to a treatment at 56°C for 30 min, rendering this plasma harmless for the cells. We thus firstly assessed the behavior of the selected bacteria in our control condition. Very surprisingly, we observed that for two *P. aeruginosa* strains (IHMA and PA7), the number of CFU diminished importantly (by 1-log) between t0 and 1h in heat inactivated plasma (Figure 28A), while the other strains were able to multiply in the same conditions. In terms of kinetic, CFUs started to decrease rapidly following the transfer in HIP, to reach a plateau after 30 min, leaving a population representing approx. 15-20 % of the initial inoculum (Figure 28B).

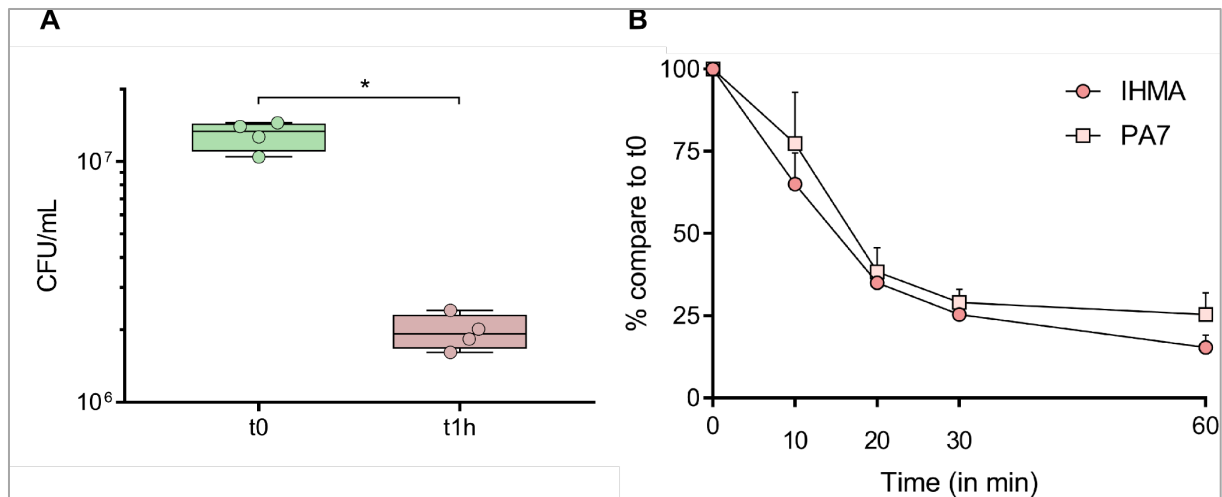


Figure 28: In heat-inactivated plasma, the number of CFUs decreases rapidly during the first hour of incubation. A) CFU/mL of the strain IHMA at t0 and t1h in heat inactivated plasma (n=4). Mann-Whitney U test: * p<0.05. **B)** Kinetics of the CFUs loss following incubation of IHMA and PA7 in HIP (n=3).

As HIP does not possess any bactericidal activity due to absence of complement, we hypothesized that the loss of CFUs was provoked by bacterial aggregation. To investigate this possibility, we visualized the strain IHMA constitutively expressing GFP in HIP. As can be observed in [Figure 29](#), in LB, prior to the incubation in HIP (t0), bacteria displayed the expected rod-shape, and were dissociated from each other, whereas in HIP, bacteria appeared as aggregates. The size of aggregates increased upon prolonged incubation, leaving no isolated single bacteria in the observed preparation [Figure 29](#).

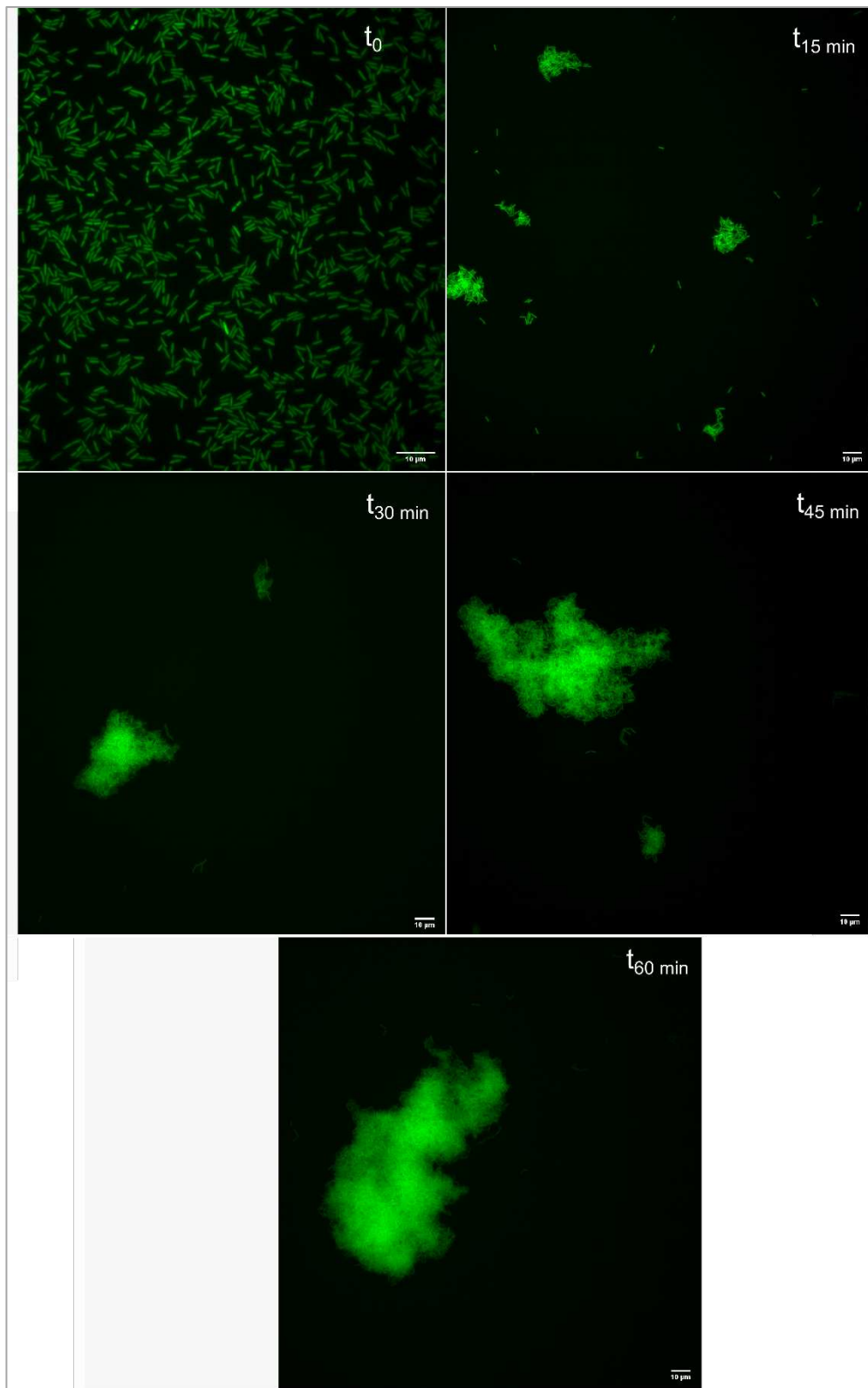


Figure 29: IHMA forms aggregates when in contact with HIP. IHMA-GFP was incubated in HIP for 1h. Each 15 min, a sample was taken to observe the state of the cells under the microscope. Magnification: 100x (t_0) and 50x (all the other time point). Scale bar represent 10 μm .

To overcome this artefact in CFU measurement, we employed a gentle sonication step (see Material and Methods) and found that it allowed to disassemble the aggregates formed after 1h in HIP, generating fully dissociated bacteria (Figure 30A). At the CFU level, mechanical disruption of the aggregates led to an increase in bacterial count by a factor 9, totally abrogating the decrease of CFU (Figure 30B). Regarding the size of these structures at 60 min, which are composed of hundreds of cells, we were expecting an increase in CFU count much more important than 1-log. We thus believe that during the serial dilutions performed before plating (see Material and Methods), aggregates are partly disassembled in smaller microcolonies.

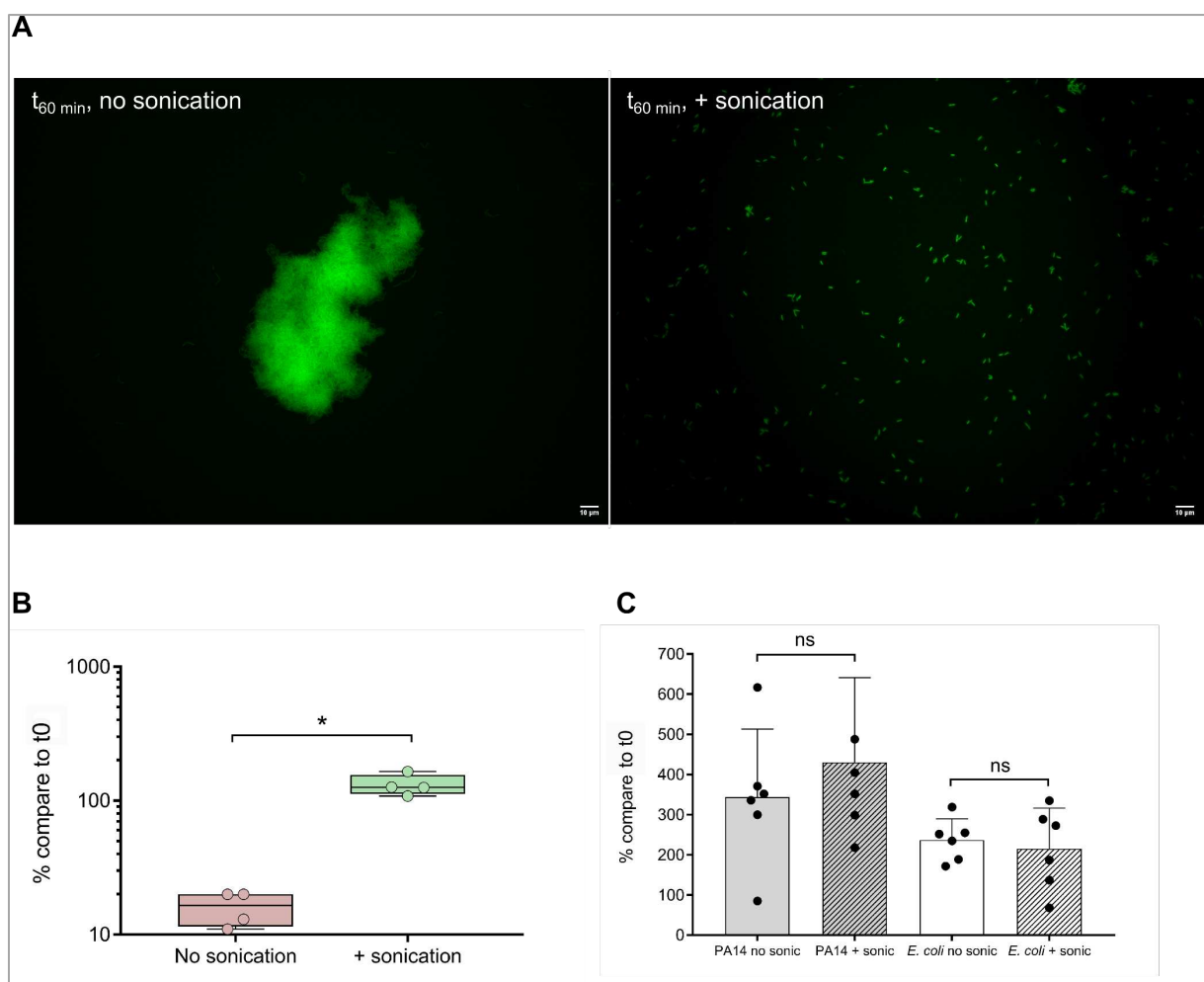


Figure 30: Sonication is an efficient way to disassemble aggregates in order to measure precisely CFU in HIP. IHMA-GFP was incubated in HIP for 1h. Then, samples were sonicated or not and observed under the microscope (magnification: 50x) (A) and plated to assess the number of CFUs compared to the initial inoculum (B) (n=4). Mann-Whitney U test: * $p < 0.05$. (C) PA14 and *E. coli* CF7968 were incubated 1h in HIP to measure the evolution of CFU compare to t_0 , with or without sonication (n=6). ns: not significant.

To ensure that the sonication does not influence bacterial viability, we tested the sonication step on strains PA14 and *E. coli* CF7968, which do not form aggregates in HIP. Both strains displayed identical colony counts regardless of the use of the sonication (Figure 30C).

We subsequently monitored the survival of our seven strains of interest in normal human plasma (non heat-treated), with or without sonication. Counterintuitively, sonication did not significantly affect the CFU measurements of the two strains IHMA and PA7 (Figure 31). Thus, aggregation does not take place in non-heated plasma, meaning that it is an artefact triggered by the treatment at 56°C. It is also possible that aggregation occurs, but that bacteria are killed by the lytic action of the complement (like IHMA which is eliminated at >99% in plasma), forming aggregates of dead bacteria which do not alter CFU measurement.

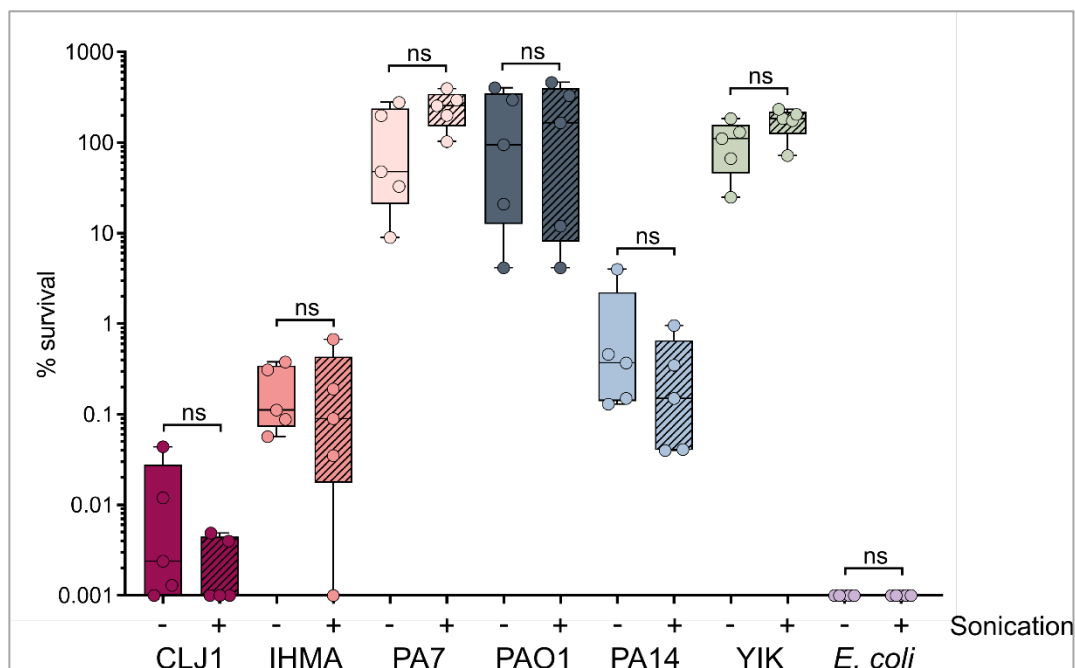


Figure 31: In normal plasma, sonication is not required to assess precisely bacterial survival. Survival in normal human plasma (% regarding the initial inoculum) of the seven strains of interest following 1h incubation, with or without sonication before plating (n=5). ns: not significant.

To further investigate the mechanism of aggregation in HIP, we observed that pre-incubation of HIP at 4°C with a high number of IHMA (2.5×10^9 CFU/mL), followed by filtering on 0.2 μm to eliminate these cells, abolished its aggregative potential upon a second incubation with the same strain, suggesting that a depletable molecule able to bind to bacteria was at the

origin of the phenomenon. Two different strategies (depletion of IgMs and mass spectrometry) were employed to identify the molecule responsible for aggregation, however without convincing results. Therefore, and because the aggregation did not impact the counts in normal plasma, this investigation was not pursued. However, we showed that aggregation could lead to a completely wrong interpretation of the CFU measurement (one could interpret the decrease in bacterial count as a loss of survival), which is solvable using gentle sonication.

Because this phenomenon did not impact the monitoring of bacterial viability in non-heated plasma, we decided that the strain's survival in whole blood and normal plasma would be measured as a percentage compared to the initial number of cells quantified at t_0 , without the use of sonication.

b) Further characterization of evaders

i) Single-cell analysis upon plasma treatment

During my EMBO short-term fellowship in the laboratory of Prof Laurence Van Melderen from the Université Libre de Bruxelles (Belgium), an expert of antibiotic persistence and single-cell analysis, I investigated whether this subpopulation of evaders shared characteristics of persisters found when bacteria are treated with some antibiotics. In original articles (Balaban et al., 2004; Goormaghtigh and Van Melderen, 2019), a microfluidic device coupled with time-lapse microscopy was used to track these rare cells and assess their growth before, during and following the antibiotic stress. As dormancy has been suggested to be involved in the formation of persister cells tolerant to antibiotics, we wondered if the same mechanism could apply to complement evaders. The microfluidic system that we used allows to perfuse culture chambers, in which bacteria have been inoculated and trapped, with the desired medium for the desired duration (Figure 32).

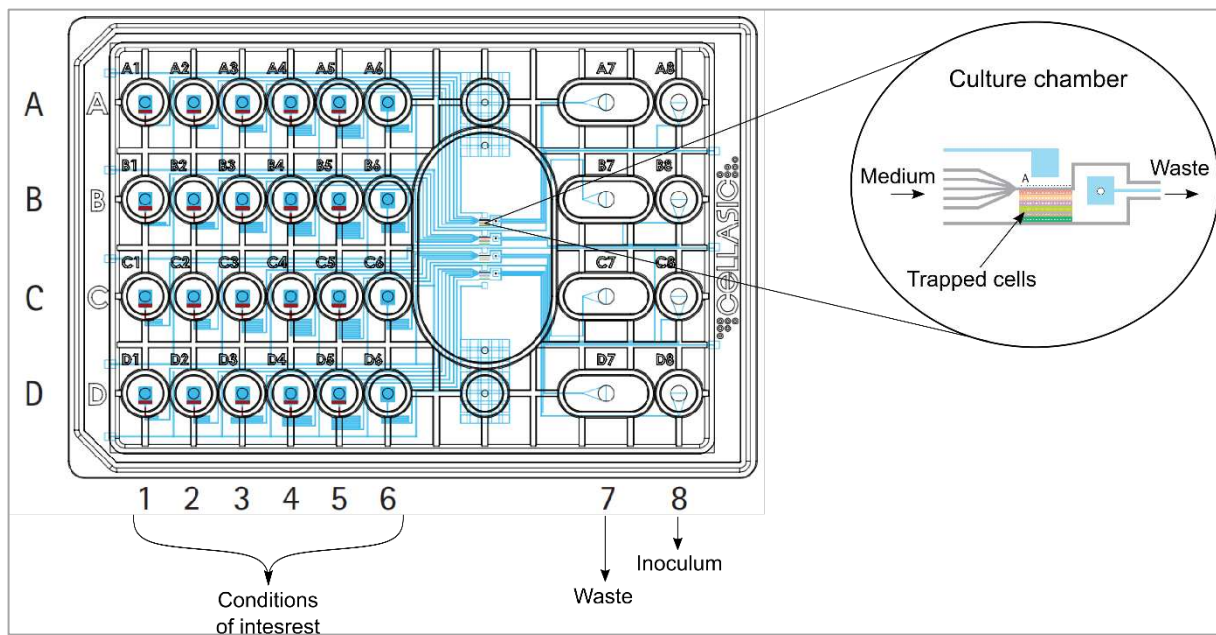


Figure 32: The microfluidic system used to perform single-cell analysis. Within the first six wells of a line, the media of interest are added in the order by which they will be used to perfuse the associated culture chamber (four culture chambers, one per line). In our case, 1: LB, 2: human plasma, 3-6: LB. In the last well (n°8), the bacterial suspension is added. The plate is sealed and linked with a controller system, which regulates the fluid movements and the perfusion of the chambers for the appropriate durations, which are previously defined by the user. Culture chambers (zoomed in) with trapped bacteria coming from well n°8 will thus receive the medium of the well n°1 for a given period, then the condition from well n°2...etc. The chambers containing the bacteria can be visualized using a microscope as their bottom is composed of a glass viewing surface, enabling live cell imaging. Bacteria are maintained immobile as they are mechanically trapped between two glass surfaces distant from 0.9 μm .

We rapidly observed that before the stress, when the microfluidic chamber was perfused with the rich culture medium LB (see Material and Methods), bacteria were not perfectly maintained motionless, making it difficult to track them. This device has already been used with a laboratory strain of *E. coli* (Goormaghtigh and Van Melderen, 2019), which does not possess any flagella or pili allowing it to swim or twitch, while the strain of *P. aeruginosa* that we used in this work (IHMA) expresses these extracellular appendages. We therefore tested the device with mutants devoid of flagella or pili ΔfliC or ΔpilA , that are respectively unable to swim or twitch. These mutants were not easier to track, showing that the motility of the bacteria was due to the continuous flow injected within the microfluidic chamber and suggesting that *P. aeruginosa* is smaller than *E. coli* and is therefore less stably caught within the microfluidic chamber. Microscopic inspection of bacterial morphology showed that following growth in LB, perfusion with plasma induced the formation of spheroplasts (Figure 33A) (red arrows).

However, we could also observe bacteria keeping a rod shape but of smaller size (white arrows). As presented on the graph, non-spheroplast bacteria in plasma are more than 40% smaller than in LB (Figure 33B). These morphological changes, probably due to permeabilization of the bacterial envelope following membrane attack complex insertion (spheroplasts) and reduced nutritious richness of the medium (cell shrinkage), rendered impossible the tracking of surviving cells during the challenge as they were not anymore trapped within the culture chamber.

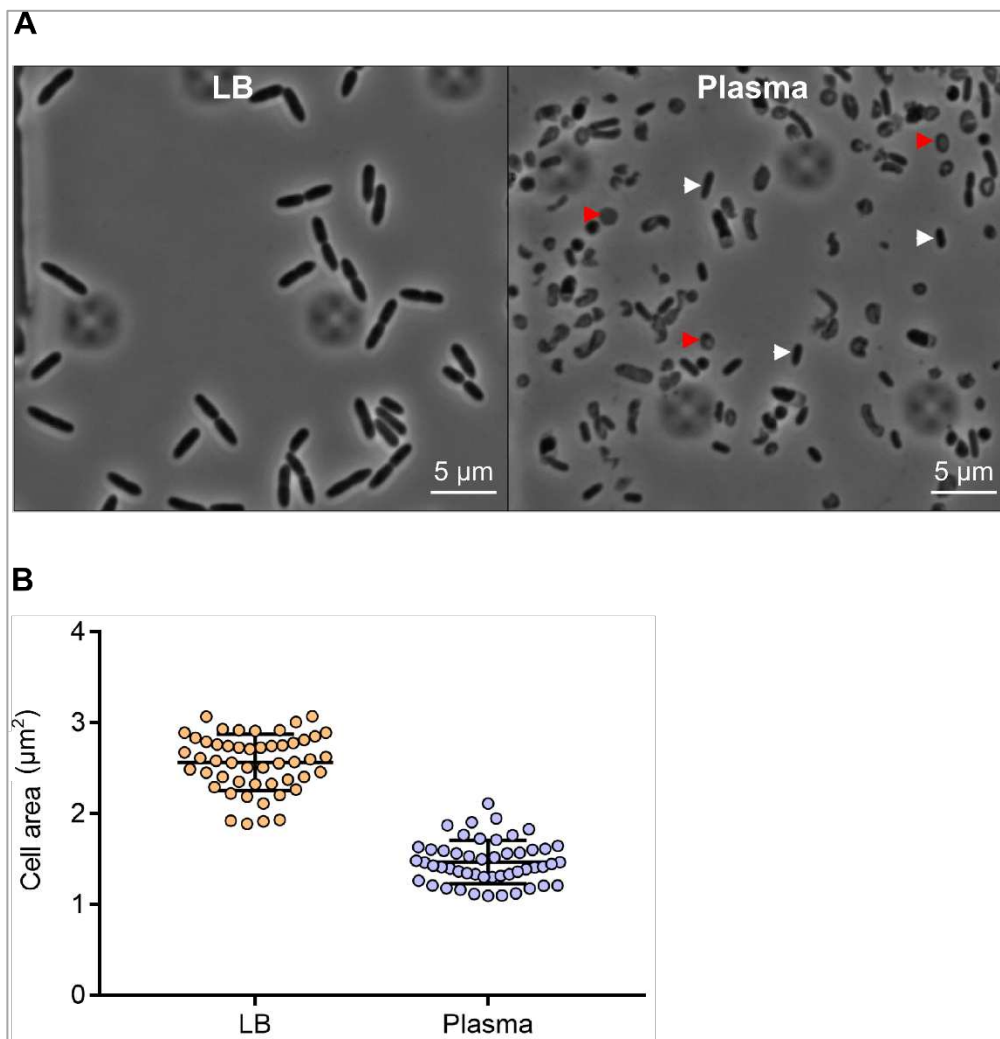


Figure 33: Bacteria treated with plasma undergo morphological changes. Following 2h incubation in LB, culture chambers with IHMA were perfused with human plasma to visualize the effect of this stress on the cells' morphology. (A) Modification of bacterial shape under the microscope, upon passage from LB to human plasma. Spheroplasts and non-spheroplast bacteria are indicated by red and white arrows, respectively. (B) Measurement of the surface of non-spheroplast cells in both conditions (n=50).

Because we did not succeed to observe the behavior of complement evaders before, during and after the challenge using the microfluidic system, we sought to identify if these rare cells were able to grow readily following stress removal or if they presented an atypical lag phase, as it has been observed for antibiotic persisters (Fridman et al., 2014). By analyzing the regrowth kinetic of single cells isolated on an agarose pad following 3h incubation in normal or heat-inactivated plasma (control condition), we could determine that complement evaders do not harbor a particular lag phase following the challenge, and start to divide rapidly upon transfer in salubrious conditions. These results were included in the research article presented in page 100 (Fig 4C-F).

ii) Stress responses triggered by the complement system

Upon antibiotic challenge, persisters arise following induction of stress responses (Goormaghtigh and Van Melderen, 2019; Peyrusson et al., 2020). As complement-evaders present persister-like features, we sought to evaluate if their tolerant phenotype could arise from a differential response to one or more major stress sensed by the cell, compared to the rest of the population.

To that end, we created reporter constructions allowing to assess the induction of the general stress response, the SOS response, the stringent response and the response induced by damages at the bacterial membrane (see Material and Methods). Promoters specifically induced upon these different stress responses were inserted upstream of the *gfp* gene. These constructions allowed monitoring:

- the general stress response: *rpoS* (induced upon entry into stationary phase, cell damage or nutrient starvation) (Whiteley et al., 2000)
- the SOS response: *sulA* (induction triggered by DNA damages) (Breidenstein et al., 2012)
- the stringent response: *uspL* (activated upon nutrient limitations and other growth-inhibiting conditions) (Boes et al., 2008)
- the envelope stress response: *algU* (Wood et al., 2006)

To verify that these constructions were functional, we exposed bacteria to conditions previously described as inducing each stress response (see Material and Methods). **Figure 34** shows that all transcriptional fusions were potently induced by the appropriate stress, with a significant increase (>2 fold) in Mean Fluorescent Intensity (MFI), between control and induced conditions.

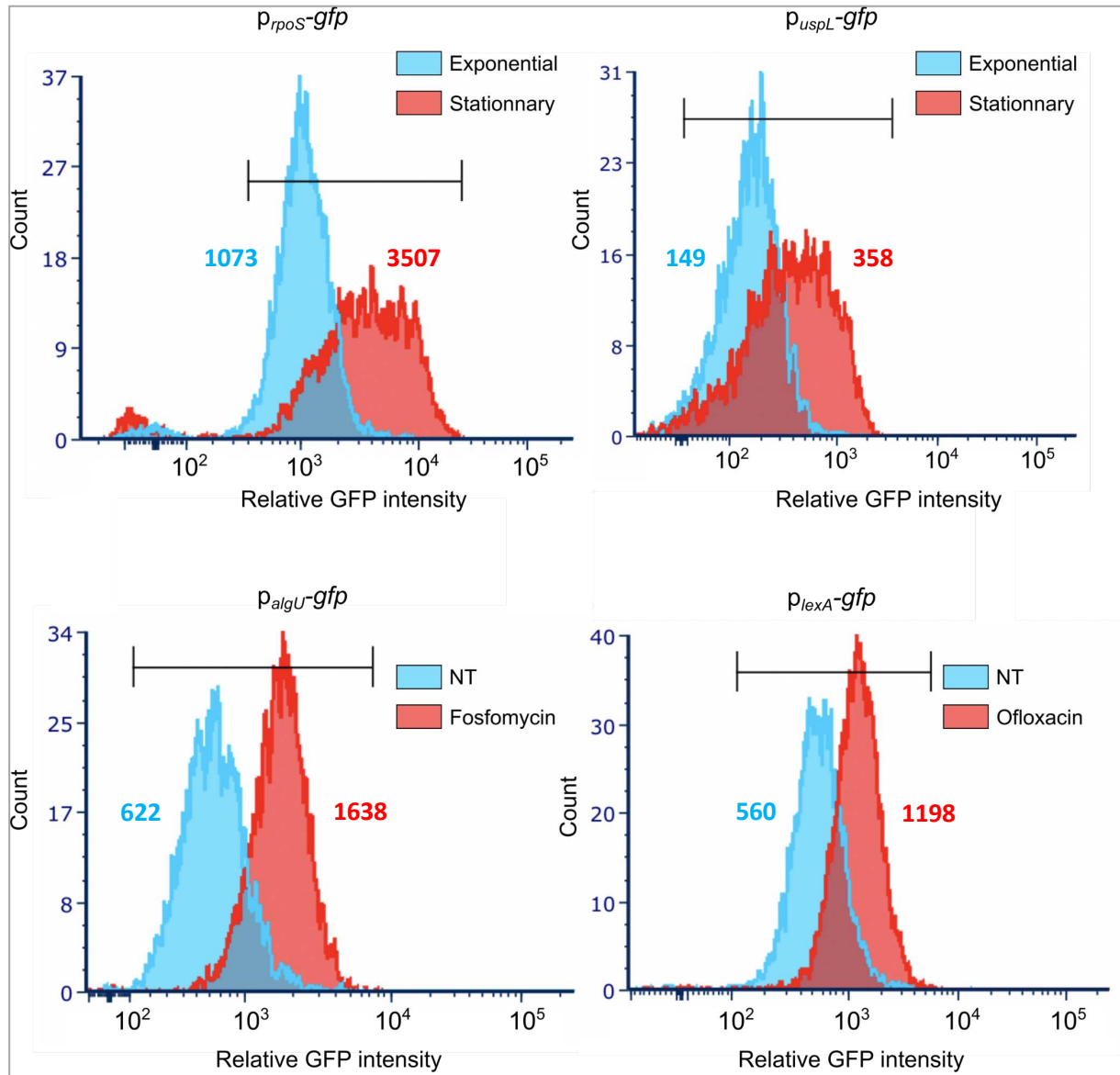


Figure 34 : Transcriptional fusions to monitor induction of stress responses are functional. PA14 carrying the indicated construction was exposed to specific stresses to assess the induction of the cognate stress response, by monitoring the GFP intensity of the cells by FACS. Fosfomycin was used at 500 $\mu\text{g}/\text{mL}$ and ofloxacin at 10 $\mu\text{g}/\text{mL}$. NT: not treated. MFIs measured for each condition (black markers) are indicated in blue (control) or in red (induced).

Following the validation of our transcriptional fusions, we assessed if complement induced one of those promoters using flow cytometry (see Material and Methods). As presented in Figure 35, incubation of the strain PA14 carrying the $p_{algU-gfp}$ on the chromosome in normal plasma for 30 min triggered an increase in GFP intensity compared to the same incubation in HIP (black marker, Mean Fluorescence Intensity: 1131 vs 790) while the activity of $p_{rpoS-gfp}$ was unchanged between the two tested conditions (black marker, MFI: 860 vs 957). The first peak on the histogram correspond to debris/dead cells which were not analyzed. Although the study of $p_{sulA-gfp}$ and $p_{uspL-gfp}$ are still underway, we will investigate whether in evaders' cells AlgU-related response differs from the rest of the population.

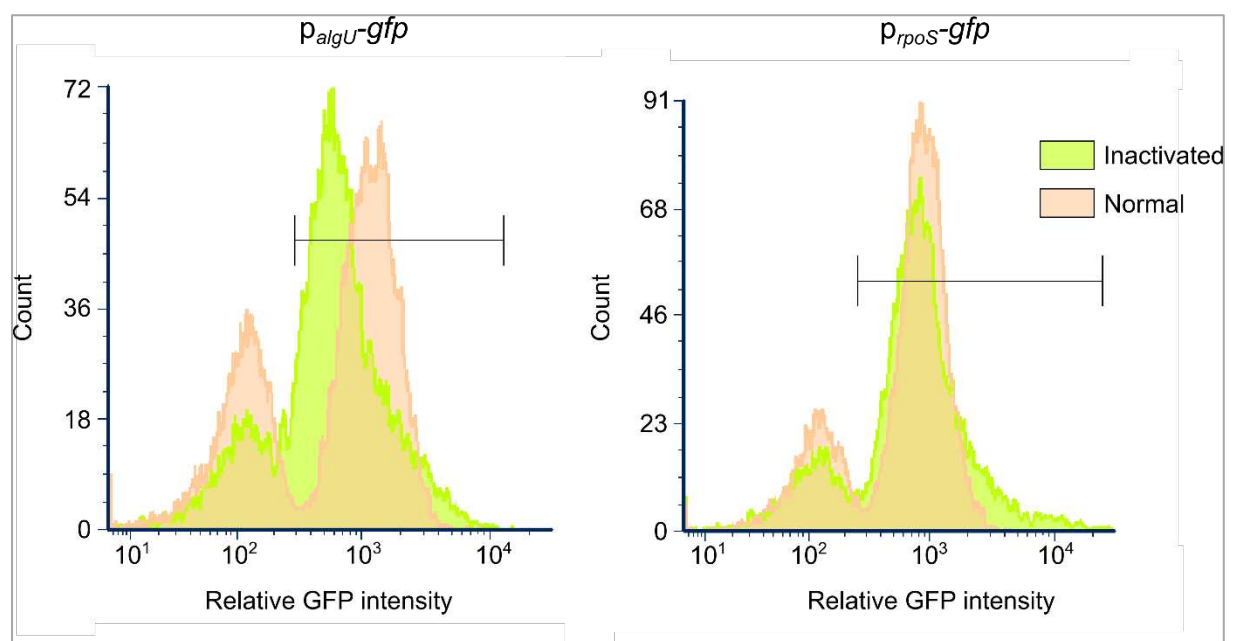


Figure 35: Incubation of *P. aeruginosa* in normal human plasma triggers the AlgU stress response. PA14- $p_{algU-gfp}$ and PA14- $p_{rpoS-gfp}$ were incubated in normal or heat-inactivated plasma for 30 min. Following the challenge, cells were analyzed by FACS to assess the GFP intensity carried by each bacterium.

c) Discussion and perspectives

Microfluidic coupled with time-lapse microscopy is a powerful tool in order to study bacterial behavior in an evolving environment at the single-cell level, and has been used to better characterize antibiotic persisters (Balaban et al., 2019; Goormaghtigh and Van Melderen, 2019). Unfortunately, the commercial device used at the Prof. Van Melderen's lab did not allow us to visualize the rare complement evaders in human plasma. Due to intrinsic smaller size (compare to *E. coli*) but mainly because of cellular morphological changes triggered by normal plasma (formation of spheroplasts or shrinkage), *P. aeruginosa* could not be maintained motionless, impairing us to track them during the entire experiment, which was the precise goal of the attempt. We believe that those modifications are due to MAC formation and insertion at the cells' surface, destabilizing the cell envelope structure. Also, shrinkage might be due to a reduction of the nutritious richness of plasma compared to the culture medium LB (e.g. availability of free iron essential for bacterial growth but sequestered by transferrin in plasma). To verify that complement activity is not the only factor influencing the morphology of the cells, the same experiment should be performed with HIP. To tackle this problem, time should be spent to optimize a microfluidic system not impacted by the reduction of the cell size. Nathan Fraikin, PhD student of the Van Melderen's lab, is currently developing this kind of system by using agarose pad which apparently better immobilize the cells while perfusing the chamber with a specific medium. However, this system still needs to be improved.

Further experiments are needed to assess whether the SOS and stringent responses are induced by the contact with active complement as observed with the AlgU stress response. The envelope stress response is activated by membrane-damaging agents (e.g. detergents, cationic antimicrobials) (Fraud et al., 2008). Upon activation, AlgU induces the expression of the multidrug efflux operon *mexCD-OprJ*, conferring resistance toward various antibiotics and the membrane-targeting biocide chlorhexidine (Fraud et al., 2008; Jeannot et al., 2008). It is thus possible that evaders harbor an earlier, stronger or continuous envelope stress response compared to the rest of the sensitive population. However, AlgU-dependent over-expression of the *mexCD-OprJ* operon in a *nfxB* mutant significantly decreased the ability of *P. aeruginosa* to withstand complement-mediated lysis in serum (Fraud et al., 2008; Martínez-Ramos et al., 2014). In line with this study, Yu and colleagues (Yu et al., 1996) observed that an *algU*-deficient mutant was less sensitive in serum compared to the parental strain, and more virulent

in a systemic model of infection. Cells with dampened AlgU-mediated stress response are thus expected to better survive in plasma. Involvement of this specific stress response in evaders' formation thus requires further investigations. Even though FACS analysis at different time points allow to determine how bacteria react in plasma, this method cannot assess the status of evaders before, during and after the challenge, which is absolutely needed to compare their behavior with their sensitive relatives.

Chapter VI

Genome-wide screen
identifies new bacterial
factors affecting the
interplay between
Pseudomonas aeruginosa and
the complement system

1) Introduction

The interaction between a pathogen and host's innate immunity dictates the success or the failure of the microorganism. Antimicrobial peptides present in high concentrations at mucosal surfaces, resident or recruited professional phagocytes at the pathogen's entry site (i.e. macrophages and neutrophils) and the complement system are major threats for invading bacteria. To cope with these effectors, pathogens have evolved protective mechanisms like LPS modifications reducing their interaction with cationic defensins, inhibition of phagocytosis by preventing actin cytoskeleton rearrangement and recruitment of negative complement regulators at their surface to resist membrane attack complex-mediated lysis (Berends et al., 2014; Ganz, 2003; Uribe-Querol and Rosales, 2017). The complement system is one of the first line of defense, acting immediately following its contact with pathogens and triggering their death either by direct lysis or by promoting their internalization by phagocytes through opsonophagocytosis. Due to its rapidity of action and its efficacy at killing bacteria (sensitive pathogens are lysed within minutes following their contact with complement), this system found in most body fluids is the major effector of the innate humoral immunity. Complement has been shown to play a pivotal role in the fight against various major human pathogens, from Gram-negative to Gram-positive bacteria, in a plethora of infectious models (DeLeo et al., 2017; Laarman et al., 2011; van der Maten et al., 2017; Mueller-Ortiz et al., 2004).

In addition to classical approaches, the serum/complement-resistome was analyzed more recently through Transposon-sequencing (Tn-Seq) screen in *A. baumannii*, *K. pneumoniae* and two *E. coli* strains (McCarthy et al., 2018; Phan et al., 2013; Sanchez-Larrayoz et al., 2017b; Short et al., 2020). These studies shed light on the complexity of the interaction between bacteria and complement, as each screen identified between 50 and 100 genes required for survival in human serum. Insertions in key genes involved in LPS biosynthesis, maintenance of membrane integrity and capsule synthesis were systematically identified, with *lpp*, *rfah* and *arnD* playing central role in complement protection of enterobacteria (McCarthy et al., 2018; Phan et al., 2013; Short et al., 2020). Global transcriptome analysis in human serum identified that in the pathogenic *E. coli* CFT073, envelope stress regulators (e.g. *rpoE* and *rcsB*) play pivotal role in the protective response against active complement (Miajlovic et al., 2014). On the other hand, genome-wide analysis performed in heat-inactivated serum, in which the

complement system is inactive, identified genes required to adapt to the nutritious conditions of the blood. Identification of mutants unable to grow in this specific environment revealed that purine and pyrimidine *de novo* synthesis is a prerequisite for growth in *Enterococcus faecium*, *P. aeruginosa* and the non-pathogenic strain *E. coli* BW25113 (Poulsen et al., 2019; Samant et al., 2008; Zhang et al., 2017). Even though common mechanisms of complement resistance were identified thanks to these studies, many genes appear to be strain specific. For instance, insertions in genes from the *tra* locus involved in pilus formation reduced survival of *E. coli* A192PP, while none of these mutants were identified when using the strain EC958 (McCarthy et al., 2018; Phan et al., 2013). More recently, Short and colleagues (Short et al., 2020) assessed the serum resistome of four *K. pneumoniae* strains, and identified only three genes (*lpp*, *rfah* and *arnD*) essential for the survival of all four strains, among the 90 genes identified as beneficial for the bacteria to persist in serum. This explains why each genome-wide screen identifies new genes influencing the interplay between pathogens and the complement system.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen responsible of deadly infections in immunocompromised and CF patients (Gellatly and Hancock, 2013). This leading cause of nosocomial infection can be responsible of a plethora of complications, including pneumoniae, keratitis, burn infections and bacteremia (Gellatly and Hancock, 2013). Two types of strains have been isolated from patients, regarding their virulence strategies. On one hand isolates expressing the type 3 secretion system (T3SS), allowing the pathogen to intoxicate eukaryotic cells with effectors ExoT, Y, S or U (Hauser, 2009). On the other hand strains harboring the two-partner secretion system ExlBA, a recently discovered type five secretion system (Elsen et al., 2014) used by the bacteria to secrete Exolysin A, a pore-forming toxin inducing lysis of different cell types (Basso et al., 2017a; Reboud et al., 2016). Both isolates use their cognate virulence factors in order to breakthrough epithelial and endothelial cell lines, allowing them to join the circulation and disseminate in the organism (Bouillot et al., 2017; Golovkine et al., 2016; Reboud et al., 2017). In the blood and in the tissues, complement has been shown to play an essential role in the fight against *P. aeruginosa* (Höpken et al., 1996; Mueller-Ortiz et al., 2004; Pont et al., 2020; Younger et al., 2003). To counteract complement-mediated killing, either through membrane attack complex (MAC)-mediated lysis or opsonophagocytosis, *P. aeruginosa* uses different strategies. Using its elastase LasB and alkaline protease AprA, this pathogen degrades key complement effectors C3 and C1q (among

others) (Bastaert et al., 2018; Hong and Ghebrehiwet, 1992; Laarman et al., 2012). LPS and exopolysaccharides (EPS) present at the surface of the bacteria impair MAC insertion within the membrane and opsonization of the cell by C3b, respectively (Goldberg and Pier, 1996; Hancock et al., 1983; Jones and Wozniak, 2017; Mishra et al., 2012; Pedersen et al., 1990; Pier et al., 2001; Thanabalasuriar et al., 2017). Finally, *P. aeruginosa* recruits negative complement regulators (e.g vitronectin, clusterin and factor H) at its surface, through the expression of Complement Regulator-Acquiring Surface Proteins Lpd and Tuf (Hallström et al., 2015, 2012; Kunert et al., 2007).

P. aeruginosa's protective factors against human complement have been exclusively identified in T3SS⁺ strains (e.g PAO1, PA14) (Dzvova et al., 2017; Hallström et al., 2015; Priebe et al., 2004). These studies often used different challenge conditions (e.g serum concentration from 5% to 80%) and different strains, making hard to assess the relative importance of each virulence factors. Even though T3SS⁺ strains represent the majority of clinical isolates (Sawa et al., 2014), strains belonging to the PA7 lineage (ExlA⁺), have also been isolated from various types of infection (Reboud et al., 2016).

We recently showed that to persist within human plasma, complement-sensitive strains of *P. aeruginosa* and other Gram-negative pathogens form complement evaders (Pont et al., 2020), rare phenotypic variants with transient tolerance toward complement. This finding raised the question of bacterial factors influencing the number of complement evaders and the mechanisms of bacterial resistance to complement attack. Here we used a Tn-Seq analysis to screen for genes either beneficial or deleterious for *P. aeruginosa* to resist complement-mediated killing. Using this powerful method of screening, we were able to identify genes previously unknown to affect the interplay between *P. aeruginosa* and the complement system in human plasma.

2) Results

a) Experimental procedure to identify genes affecting the interplay of bacteria with the complement system

This screen relies on the use of a transposon (Tn) mutant library created using the clinical isolate IHMA879472 (hereafter IHMA) that lacks T3SS but secretes Exolysin A (Kos et al., 2015; Reboud et al., 2016). The library was constructed by using a random mariner-based Tn mutagenesis (Goodman et al., 2009; Skurnik et al., 2013) during which a mariner Tn inserts stochastically at a TA site on the bacterial genome thanks to the action of the Mariner C9 transposase. This procedure allowed the generation of a saturated library of approx. 300.000 unique mutants, each harboring a Tn inserted at a different site in its genome. The insertion of the transposon within the genome of the cell triggers two possible outcomes. Either the disruption of the coding sequence in which it inserts, abolishing the expression of the gene, or it can enhance the expression of downstream elements, thanks to an internal promoter (*P_{tac}*) present in its sequence.

As the parental strain IHMA is sensitive to complement-mediated lysis (Pont et al., 2020), this screen is based on a gain of function, as mutants of interest will present an increased fitness in plasma. The library was incubated 3h in LB or in pooled human plasma, as described in Material and Methods. Tn-mutants with increased resilience to complement compared to WT were selected in the output (Figure 36). Following the first incubation, remaining Tn-mutants in both conditions were allowed to multiply in LB and were then identified thanks to Illumina sequencing, allowing determining the proportion of each mutants in both the input and the output.

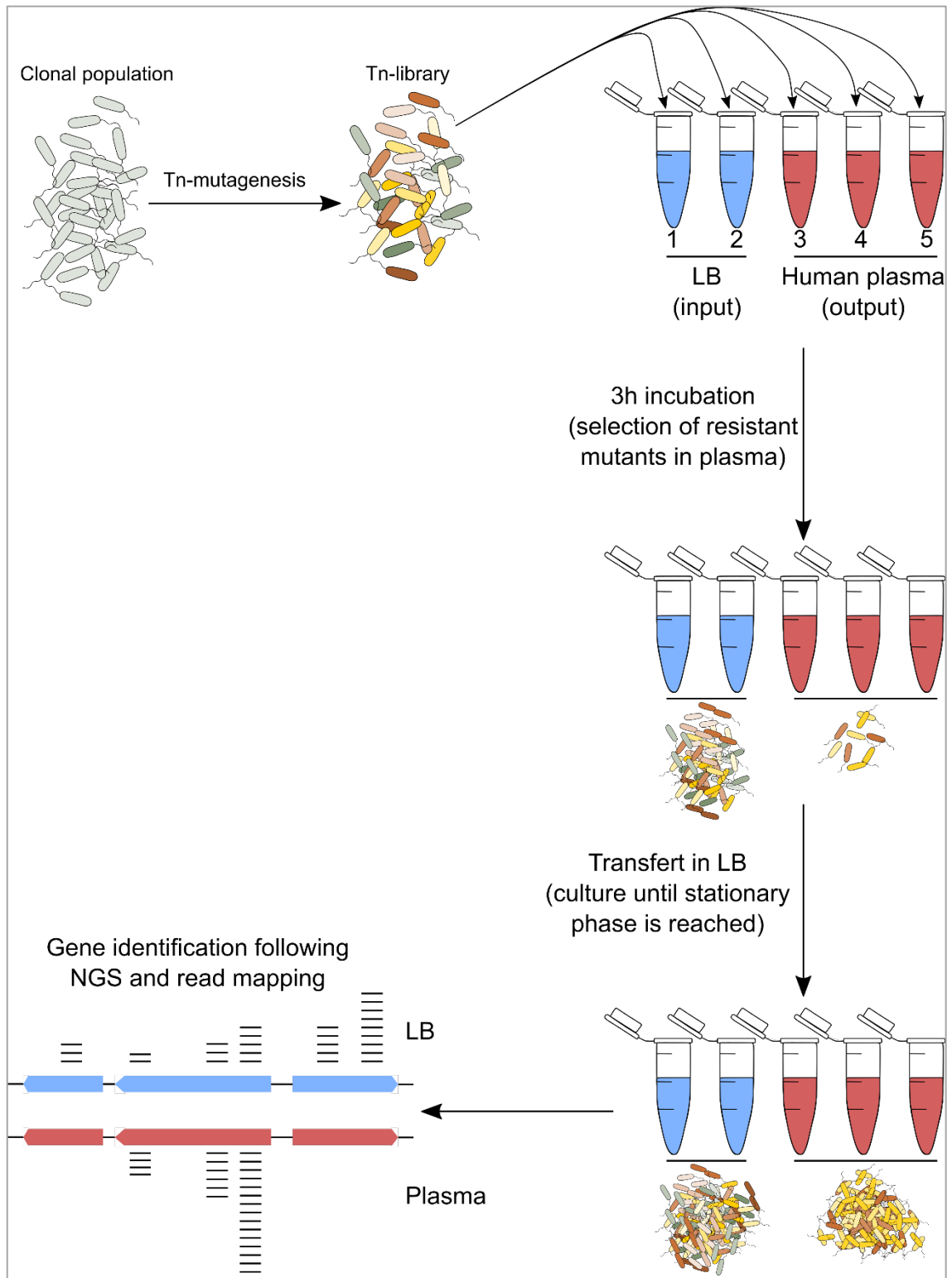


Figure 36: Design of the Tn-seq screen performed in a pool of human plasma using a Tn-mutant library generated in the complement-sensitive strain IHMA.

b) Tn-seq identifies known and unknown players of complement resistance

The quality of the library was assessed by analyzing the reads obtained from the “input” in LB. As shown in Figure 37, Tn insertions were found all along the genome, targeting 89% of the genes. Among these coding sequences, the average TA occupation was 68%, meaning that most of the genes were targeted at multiple positions. Except three genes (*fimV*, *cyaB* and *pilJ*), which yielded reads representing between 1.1 and 1.4% of the total read count, no Tn insertion was over-represented in the input. Absence of reads in LB was observed for 692 genes, meaning that their disruption following Tn insertion was lethal. This number of essential genes in rich medium correlates with the one determined by Poulsen and colleagues (Poulsen et al., 2019), who identified that the size of the essential genome in LB ranged from 388 to 721 genes, depending of the *P. aeruginosa* strain.

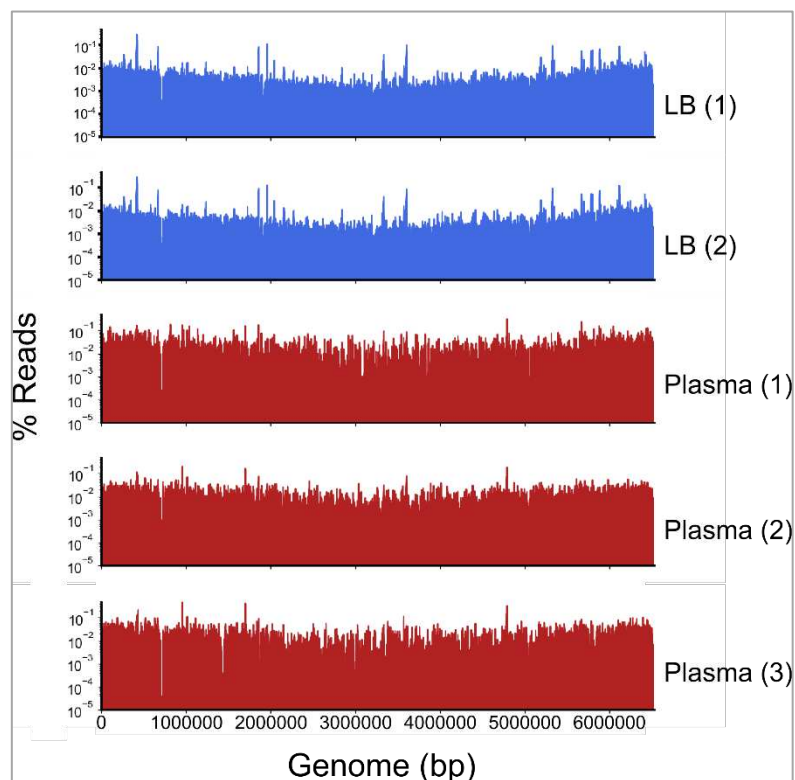


Figure 37: Characterization of the IHMA transposon library. Overview of the positions of every insertion detected in each sample, along with their frequency among the entire population. Note the highly similar profiles of the two input replicates, and the presence of common high-frequency peaks between the three outputs.

Comparison of the read counts (see Material and Methods) between the input and the output conditions (replicates are pooled for the analysis) revealed insertions in 18 genes (Figure 38A) and 9 intergenic regions (Figure 38B) that triggered a significant increase in survival in plasma (Table 3). Insertions in 34 coding and 10 intergenic regions were found with significantly lower abundance following the challenge.

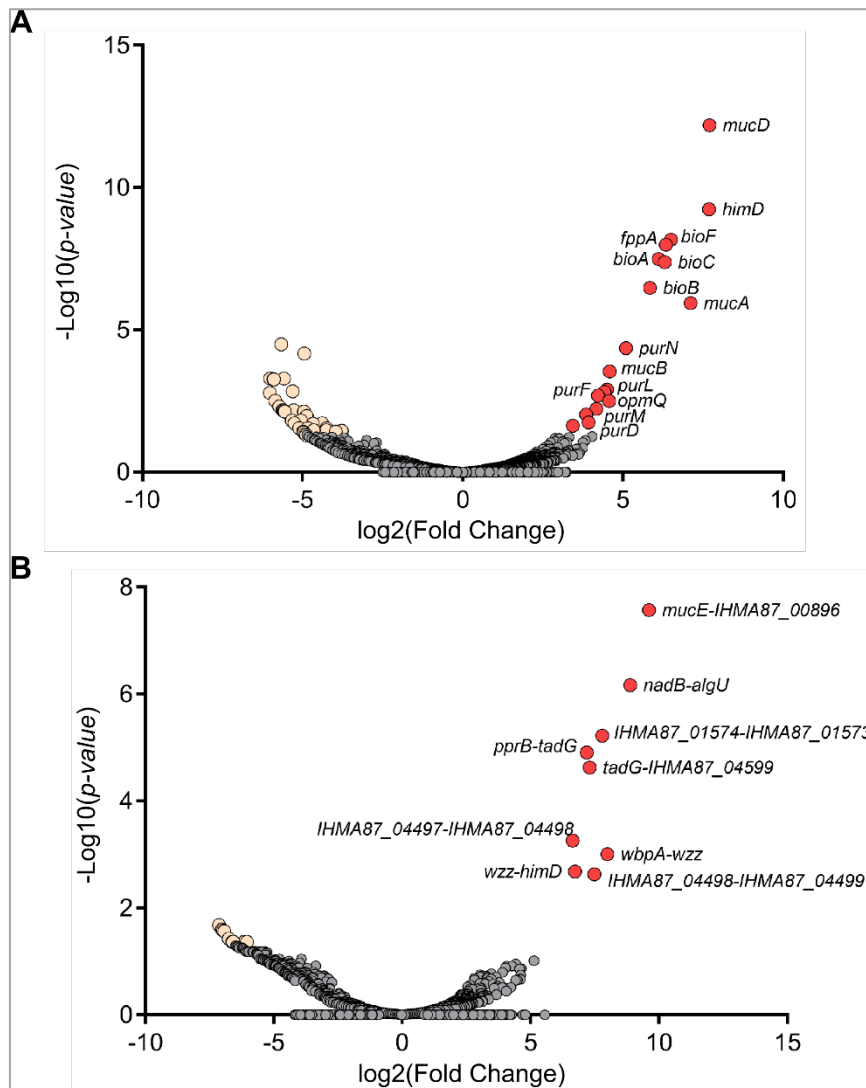


Figure 38: Analysis of the screen for increased resistance to plasma of the IHMA Tn library. Volcano plots showing the insertions in coding (A) or intergenic (B) regions significantly enriched (red) following the challenge in plasma. In beige, hits with significantly lower frequency in the output.

	Homologues (PAO1)	Gene name (PAO1)	Log2 fold increase	Adj p-value
Coding regions				
<i>IHMA87_04490</i>	<i>PA0766</i>	<i>mucD</i>	7,7	6,4E-13
<i>IHMA87_01786</i>	<i>PA3161</i>	<i>himD</i>	7,7	5,8E-10
<i>IHMA87_00477</i>	<i>PA0501</i>	<i>bioF</i>	6,5	6,7E-09
<i>IHMA87_04596</i>	<i>PA4295</i>	<i>fppA</i>	6,3	1,0E-08
<i>IHMA87_00400</i>	<i>PA0420</i>	<i>bioA</i>	6,1	3,2E-08
<i>IHMA87_00479</i>	<i>PA0503</i>	<i>bioC</i>	6,3	4,2E-08
<i>IHMA87_00476</i>	<i>PA0500</i>	<i>bioB</i>	5,8	3,4E-07
<i>IHMA87_04493</i>	<i>PA0763</i>	<i>mucA</i>	7,1	1,1E-06
<i>IHMA87_04292</i>	<i>PA0944</i>	<i>purN</i>	5,1	4,3E-05
<i>IHMA87_04492</i>	<i>PA0764</i>	<i>mucB</i>	4,6	2,9E-04
<i>IHMA87_01837</i>	<i>PA3108</i>	<i>purF</i>	4,5	1,3E-03
<i>IHMA87_01191</i>	<i>PA3763</i>	<i>purL</i>	4,4	1,5E-03
<i>IHMA87_02764</i>	<i>PA2391</i>	<i>opmQ</i>	4,2	2,0E-03
<i>IHMA87_04595</i>	<i>PA4294</i>	<i>PA4294</i>	4,6	3,1E-03
<i>IHMA87_05548</i>	<i>PA5066</i>	<i>hisI</i>	4,2	5,9E-03
<i>IHMA87_04291</i>	<i>PA0945</i>	<i>purM</i>	3,8	9,4E-03
<i>IHMA87_05343</i>	<i>PA4855</i>	<i>purD</i>	3,9	1,8E-02
<i>IHMA87_05201</i>	<i>PA4726</i>	<i>cbrB</i>	3,4	2,3E-02
Intergenic regions				
<i>IHMA87_00895-IHMA87_00896</i>	<i>PA4032-PA4033</i>	<i>PA4032-mucE</i>	7,8	2,2E-04
<i>IHMA87_04494-IHMA87_04495</i>	<i>PA0761-PA0762</i>	<i>nadB-algU</i>	9,1	2,2E-04
<i>IHMA87_01573-IHMA87_01574</i>	<i>PA3368-PA3369</i>	<i>PA3368-PA3369</i>	7,5	2,9E-04
<i>IHMA87_04597-IHMA87_04598</i>	<i>PA4296-PA4297</i>	<i>pprB-tadG</i>	7,7	2,9E-04
<i>IHMA87_04598-IHMA87_04599</i>	<i>PA4297-PA4298</i>	<i>tadG-PA4298</i>	7,1	4,7E-04
<i>IHMA87_04497-IHMA87_04498</i>	<i>PA0759-PA0760</i>	<i>PA0759-PA0760</i>	8,7	9,1E-04
<i>IHMA87_01787-IHMA87_01788</i>	<i>PA3159-PA3160</i>	<i>wbpA-wzz</i>	7,4	1,7E-03
<i>IHMA87_01786-IHMA87_01787</i>	<i>PA3160-PA3161</i>	<i>wzz-himD</i>	7,2	1,4E-02
<i>IHMA87_04498-IHMA87_04499</i>	<i>PA0758-PA0759</i>	<i>PA0758-PA0759</i>	5,8	3,2E-02

Table 3: Genomic regions with Tn insertion significantly enriched following challenge in plasma.

Analysis of significant hits revealed several genomic regions encoding regulators of alginate synthesis. Disruption of genes coding for negative regulators MucD and MucA lead to overproduction of these exopolysaccharides, while insertion in intergenic regions upstream of positive regulators of the same pathway *algU* and *mucE* induce an over-expression of these genes, increasing the biosynthesis of alginates (Figure 39). Alginate is an exopolysaccharide interfering with complement binding and activation at the bacterial surface when acetylated, explaining why these insertions increase the mutants' survival (Pedersen et al., 1990; Pier et

al., 2001). Insertions upstream of the O-antigen biosynthetic gene cluster, like in *himD* (Figure 38A) or in the two intergenic regions *wzz-himD* and *wbpA-wzz* (Figure 38B) likely increase the expression of key proteins involved in LPS synthesis. Wzz is responsible of the generation of long-OSA repeats, already shown to play an essential role in the defense of *P. aeruginosa* against complement (Kintz et al., 2008). Finally, we observed several insertions disrupting the *tad* locus, involved in the biosynthesis of the Type IVb pili, an appendage involved in bacterial attachment on abiotic surfaces and host cells. The gene *fppA* encodes a prepilin peptidase ensuring the maturation of the the Flp prepilin, the major component of the pilus (Bernard et al., 2009). *pprB* is a positive regulator of the *tad* locus assembling the adhesive appendage (Bernard et al., 2009). Type IV pili have already been shown to contribute to serum-resistance in *V. cholerae* (Chiang et al., 1995) and to recruit the negative complement regulator C4BP which bind to the N-terminus of PilC in *N. gonorrhoea* (Blom et al., 2001).

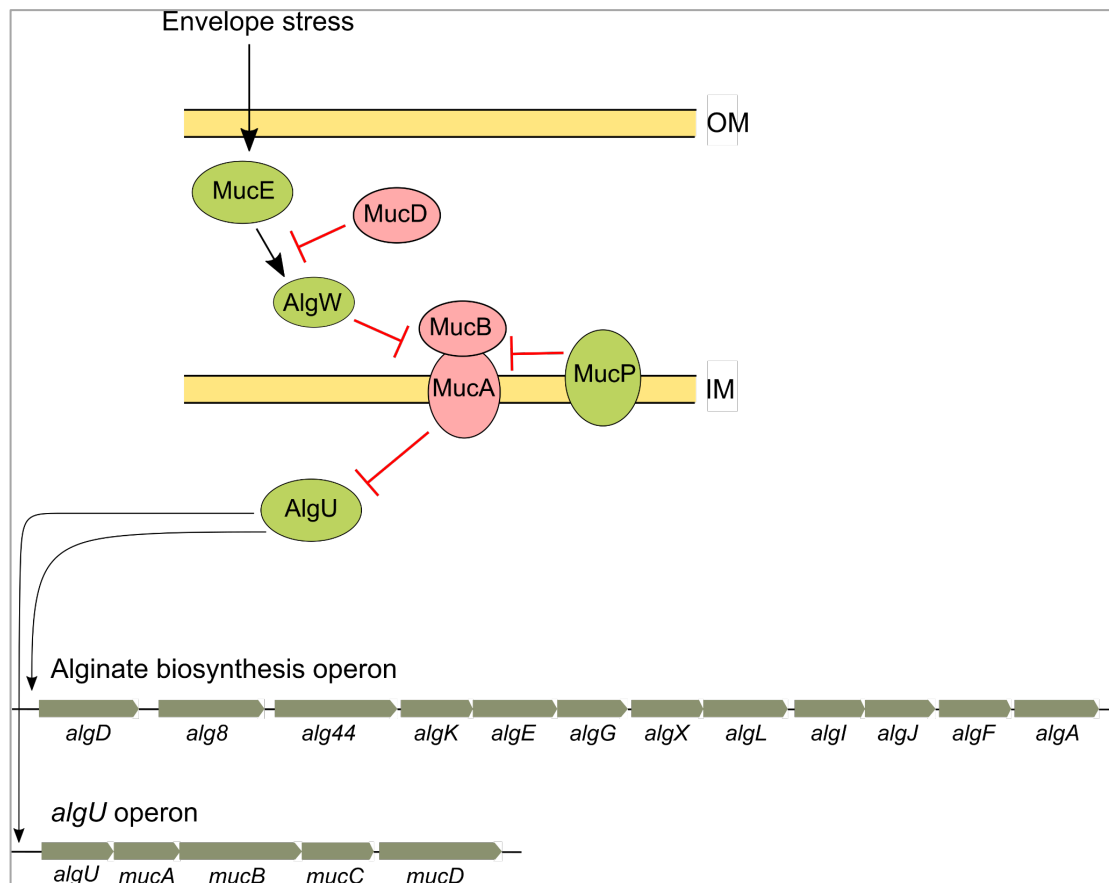


Figure 39: Alginate biosynthesis regulation. In steady state, MucA sequesters the sigma factor AlgU at the membrane. MucB stabilizes this complex. Upon envelope stress sensing, MucE activates the proteolytic activity of AlgW. MucA is then cleaved by AlgW and MucP, freeing AlgU and allowing it to activate the transcription of the alginate biosynthesis operon leading to alginate production. AlgU also activates its own transcription. MucD is another negative regulator of alginate synthesis, acting by preventing the AlgW activation by MucE.

Tn-seq analysis revealed that many genes involved in the biotin (*bioF*, *bioA*, *bioC*, *bioB*) or purine (*purN*, *purL*, *purF*, *purM* and *purD*) biosynthesis pathways, located at different positions on the genome, were disrupted in mutants significantly enriched in the output (Figure 38A and 40, Table 3). Finally, a Tn insertion within the gene coding for the outer membrane protein OpmQ, involved in the recycling of pyoverdine (Imperi et al., 2009), was detected as a significant hit.

In order to confirm these results, we engineered a single-gene knockout in the reference strain PA14, which displays similar killing kinetics as IHMA in plasma. We first inactivated *bioB* and *purD* as they encode key enzymes (the biotin synthase and the phosphoribosylamine—glycine ligase) of the biotin and purine synthesis pathways, respectively. Mutants were incubated in human plasma for three hours, and their survival compared to the parental strain was measured every hour by CFU counts as described in Material and Methods. We observed that after 1h in plasma, mutants defective in biotin and purine synthesis displayed a survival rate 6 and 50 times higher than their parental strain, respectively, however with survival reaching WT levels at 3 h (Figure 41). This suggests that their survival in plasma differs depending if they are incubated alone or in presence of other Tn-mutants resistant to the action of the complement, as it was already described in the case of pathogenic *E. coli* (Phan et al., 2013). For instance, Tn-mutants with increased production of alginates will secrete these exopolysaccharides in the surrounding medium, which can then benefit to other cells already more resistant compare to the parental strain, like Tn::*bioB* and Tn::*purD*.

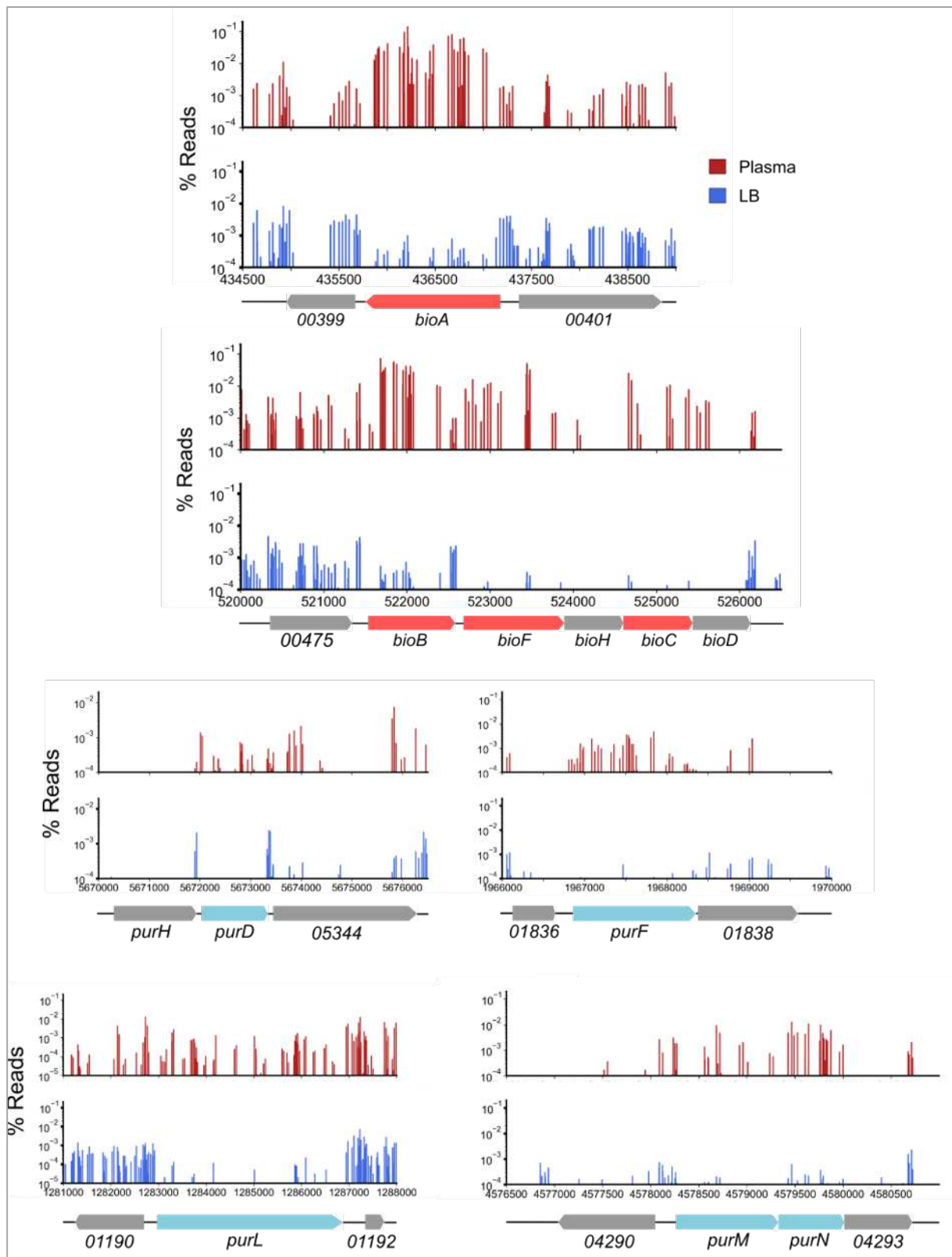


Figure 40: Mutants with insertions in genes controlling biotin and purine biosynthesis are recurrently enriched following the challenge in plasma. Detailed view of the coding regions with occupied TA affecting these two pathways and the incidence of each insertion within the total population in the input and the output.

The mutant $\Delta opmQ$ showed no difference compared to the wild type strain showing that this hit might be a false positive. Indeed, genome-wide screening is prone to generate false positive hits, as reported by Phan and colleagues (Phan et al., 2013) who could not experimentally confirm 15% of their Tn-seq hits. Alternatively, the involvement of OpmQ in the interplay with the complement system is likely to be strain specific.

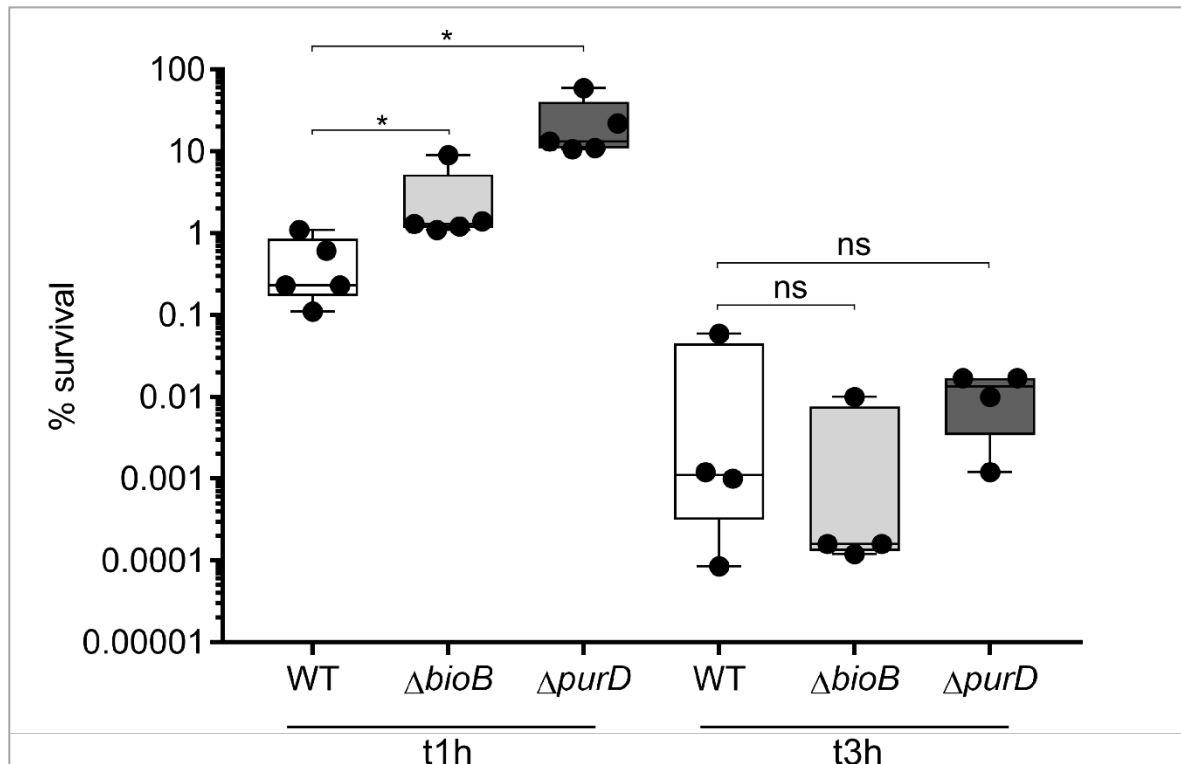


Figure 41: Targeting the biotin and purine biosynthesis pathways increases complement-resilience during the first hour of incubation. PA14 WT, $\Delta bioB$ and $\Delta purD$ were challenged for 3h in human plasma to evaluate their survival all along the incubation. Mann-Whitney U test: * $p < 0.01$. ns: non-significant.

One of the top hits following the screen, with 181-fold enrichment in the output (Table 3, Figure 42A), was the intergenic region between *IHMA87_01573* and *IHMA87_01574* (Figure 38B). Two different insertion mutants were isolated during the screen, re-sequenced after semi-random PCR and conserved for direct analysis. These isolates harbored the transposon inserted in this intergenic region and sequence analysis positioned their insertions at 11 and 13 nucleotides upstream of the ATG start codon of the gene *IHMA87_01573*, annotated as of unknown function on Pseudomonas genome project. We validated the

phenotype of these mutants in plasma where their survival was increased by >3logs compared to the parental strain, following 3h incubation (Figure 42B and C).

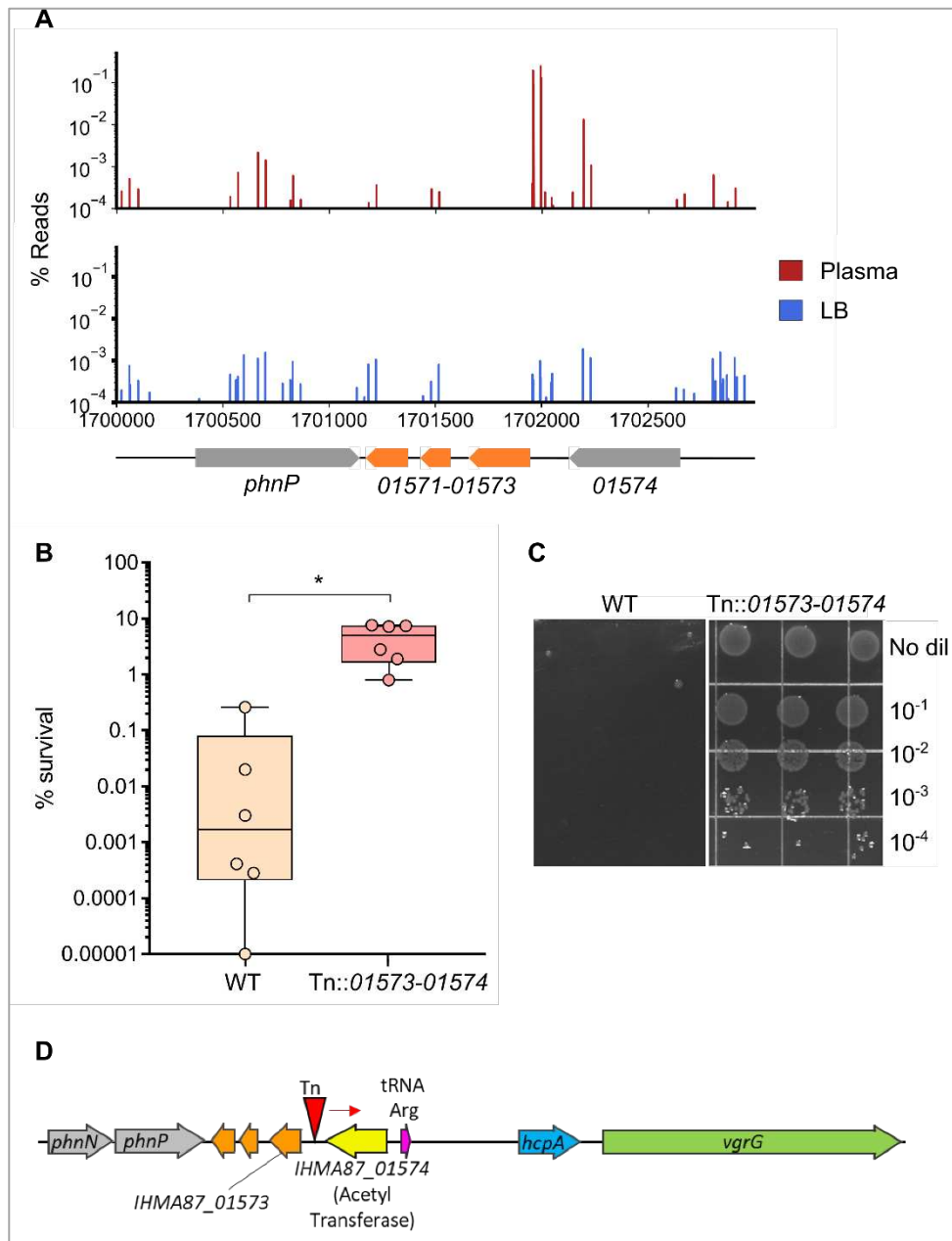


Figure 42: Tn Insertion in the intergenic region *IHMA87_01573-IHMA87_01574* increases survival in plasma. (A) Detailed view of the intergenic insertions highly enriched in the output. (B) and (C) Incidence of the presence of a transposon upstream of the gene *IHMA87_01573* on the mutant's survival after 3h in plasma, compared to the WT strain. Mann-Whitney U test: * p<0.001. (D) Genetic environment around the insertion and precise localization of the Tn in the mutant of interest. The red arrow corresponds to the orientation of the Tn internal promoter *P_{tac}*.

As the transposon is inserted in an intergenic region between genes *IHMA87_01573-IHMA87_01574*, its effect could not be directly predicted. Indeed, the internal transposon *Ptac* orientated towards the acetyltransferase encoding gene *IHMA87_01574*, possibly interferes with its transcription (Figure 42D). Alternatively, the Tn insertion in the putative promoter region of the predicted operon *IHMA87_01573-IHMA87_01571*, could up or down regulate the level of its expression. Therefore, we undertook the construction of several deletion mutants. The created mutants for the genes *IHMA87_01573* and *IHMA87_01574* in IHMA behaved in plasma as the wild type (Figure 43), suggesting that the beneficial effect of the insertion in the Tn-mutant was not due to inactivation of the either gene.

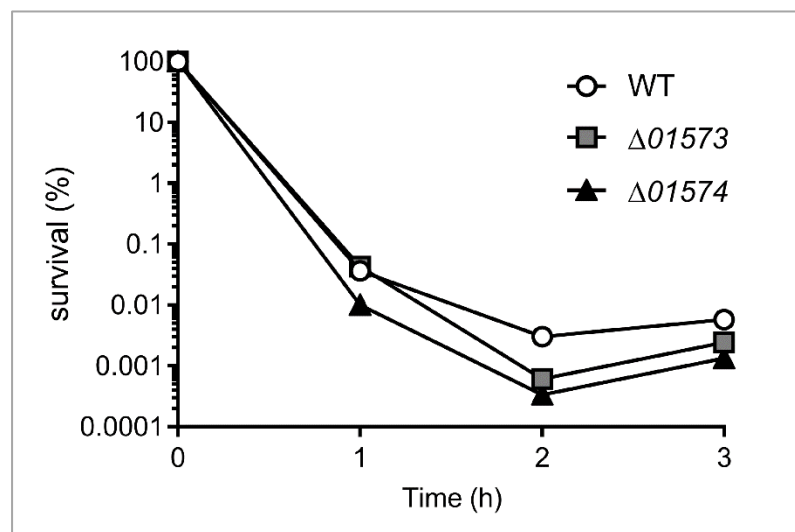


Figure 43: Deletion of the two genes *IHMA87_01573* or *IHMA87_01574* does not influence the survival of IHMA in plasma. Kinetic survival of IHMA WT, $\Delta IHMA87_01573$ and $\Delta IHMA87_01574$ in human plasma for 3h, with CFU measurement performed every hour (n=1).

To assess the genome-wide impact of the Tn::*01573-01574* insertion, we performed RNA-Seq on IHMA and IHMA-Tn::*01573-01574* mutant in replicates (experiments and analysis were done by Manon Janet-Maître, 2nd year PhD student in the Team). All three genes *IHMA87_01571*, *01572* and *01573*, were overexpressed 20 times compared to WT (Table 4). In addition, genes within *algU* operon and *algR*, a positive regulator of the alginate biosynthesis operon, were slightly but significantly over-expressed (>2 times) in the mutant (Table 4). Upregulation of alginate biosynthetic pathway explains the “mucoïd” phenotype of the Tn mutant observed on LB plate (Figure 44). Because over-expression of the previously uncharacterized *IHMA87_01573-IHMA87_01571* operon led to increased tolerance to complement, we named these genes “serum resistant genes” (*srgA*, *srgB*, *srgC*). The first

protein SrgA encoded by the operon is predicted to carry an N-terminal cleavable Sec-signal peptide, meaning that it might be translocated to the periplasm through the Sec machinery. SrgB and SrgC are predicted to be localized at the inner membrane. It is possible that *srgABC* gene products are new positive regulators of alginate biosynthesis.

Genes	Homologues (PAO1)	Gene name (PAO1)	Log2 fold increase	Adj p-value
<i>IHMA87_01571</i>	<i>PA3369</i>		4,6	7,0E-287
<i>IHMA87_01572</i>	<i>PA3371</i>		4,7	1,9E-133
<i>IHMA87_01220</i>	<i>PA3370</i>		4,3	3,7E-133
<i>IHMA87_00707</i>	<i>PA0763</i>	<i>mucA</i>	1,7	1,5E-92
<i>IHMA87_00714</i>	<i>PA0762</i>	<i>algU</i>	1,5	7,5E-66
<i>IHMA87_05665</i>	<i>PA0764</i>	<i>mucB</i>	1,4	3,5E-59
<i>IHMA87_00717</i>	<i>PA5261</i>	<i>algR</i>	1,3	2,5E-43
<i>IHMA87_06070</i>	<i>PA0766</i>	<i>mucD</i>	1,1	5,3E-42

Table 4: Genes from the operons *IHMA87_01573-IHMA87_01571* and *algU* are overexpressed in the *Tn::IHMA87_01573-IHMA87_01574* mutant. Results were obtained by RNA-Seq (Janet-Maître, unpublished).



Figure 44: Tn insertion upstream of the *srg* operon triggers the apparition of a mucoid phenotype. Serial dilutions in PBS of the two backgrounds were plated and incubated at 37°C for 36h to compare their aspect. Note the shiny, mucoid appearance of the mutant *Tn::srg*.

3) Discussion and perspectives

The genome-wide screen (Tn-seq) allowed identifying new factors affecting the survival of *P. aeruginosa* (strain IHMA87) in the presence of complement. We found that many mutants affected in *bio/pur* displayed an increased fitness in plasma/complement resistance.

Purine biosynthesis is central for the general metabolism of the bacterial cell, as it allows the generation of the essential AMP and GMP nucleotides. Auxotrophic mutants thus rely on the acquisition of purines from their surrounding environment, and have been shown to be more sensitive to a wide variety of stresses and less virulent (Poulsen et al., 2019; Yee et al., 2015). The reason why these Tn-mutants appear to better survive in plasma is enigmatic, and needs further experiments to emit consistent hypothesis. For instance, an RNA-Seq on the $\Delta purD$ background in LB could provide information on the dysregulated genes in this mutant, some of them possibly directly involved in the interaction with the complement. Knowing that purine biosynthesis is essential for pathogens to grow in serum without active complement (Poulsen et al., 2019; Samant et al., 2008), bacteria unable to *de novo* synthesize purines are expected to be metabolically inactive in plasma. The mechanism by which MAC kills bacteria is still not well understood (Berends et al., 2014). Decades ago, it was hypothesized that complement-mediated killing of Gram-negative bacteria was an energy-dependent process, requiring the generation of bacterial ATP (Taylor and Kroll, 1983). Bacteria with defective purine biosynthesis pathway in plasma might thus benefit from this metabolic dormancy to withstand MAC-mediated killing. However, our previous results (Pont et al., 2020) have shown that metabolically inactive cells are hypersensitive in plasma. Biotin (vitamin B7 or vitamin H) is a cofactor of enzymes involved in essential pathways like amino acid and fatty acid metabolism. The positive role of biotin in bacterial virulence was described for *Francisella novicida* and *Mycobacterium tuberculosis*, which allows the bacteria to multiply within macrophages (Feng et al., 2015; Salaemae et al., 2016). Very recently, the first study on the role of biotin synthesis in the pathogenicity of *P. aeruginosa* was conducted (Carfrae et al., 2020). The authors observed that like for the purine biosynthesis pathway, auxotrophic mutants presented a growth defect in human plasma and attenuated virulence in systemic mice infection. However, the PAO1 $\Delta bioA$ strain used was complement resistant, meaning that the decreased pathogenicity is not due to increased sensitivity to membrane attack complex. Molecular

mechanisms leading to increased resilience toward complement-mediated lysis by bacteria deficient in biotin synthesis is unknown, but again the reduced metabolic activity could be a cause. It is interesting to note that biotin auxotrophic mutants have never been identified as attenuated from Tn-seq in mice models of infection (Carfrae et al., 2020). Indeed, the concentration of circulating biotin is 40 times higher in mice than in human, allowing biotin-deficient mutants to uptake sufficient amount of biotin and sustain normal growth (Carfrae et al., 2020).

To further validate Tn-seq results regarding the beneficial effect of disrupting the purine and biotin synthesis pathways, the deletion mutants should be constructed in the IHMA strain, which was originally used for the screen. Also, the capacity of a mutant over-expressing alginates to cross-protect $\Delta bioB$ or $\Delta purD$ mutants in plasma should be addressed, to explain the discrepancies observed between the Tn-seq results after 3h in plasma and the fact that the two deletion mutants lost their phenotype at 3h.

In addition to Tn insertions negatively affecting the purine and biotin synthesis pathways, this screen allowed us to identify an uncharacterized operon with three genes whose overexpression increased bacterial survival in plasma up to 1000 fold. Overexpression of these ‘serum resistant genes’, or ‘*srg*’, seemed to positively affect alginate synthesis by this Tn-mutant, likely responsible for its heightened ability to cope with the complement system. Confirmation that the product(s) of the *srg* genes are responsible for this increased survival will be performed by deleting the *srg* operon in the Tn-mutant of interest. Experiments aiming at better understanding the molecular mechanisms underlying this strong phenotype are undergoing. Notably, the capacity of the mutant to activate the three complement pathways, as well as its ability to bind C3b and MAC will be assessed. Interestingly, the overexpression of SrgABC did not increase antibiotic resistance compare to parental strain (Minimal Inhibitory Concentrations measured in carbenicillin, tobramycin, ofloxacin and polymyxin B). In addition, the virulence of this mutant in the *Galleria melonella* model of infection will be assessed.

Chapter VII

Inflammasome activation
by *Pseudomonas aeruginosa*'s
ExlA pore-forming toxin is
detrimental for the host

1) Research Article

Stéphanie Bouillot, Stéphane Pont, Benoit Gallet, Christine Moriscot, Vincent Deruelle, Ina Attrée, Philippe Huber (Cellular Microbiology (2020))

Inflammasome activation is an efficient mechanism involved in the defense of the host against various pathogens. It allows to alert the immune system from the presence of foreign invaders, and permit the proper development of the inflammatory response, enabling the recruitment and activation of leukocytes to the site of infection, a prerequisite for bacterial clearance. *P. aeruginosa* is known to activate the NLRC4 system through its type three secretion system, and the NLRP3 inflammasome thanks to the action of the pore-forming toxin Exolysin A, secreted by a subset of strains. It has been shown that in the particular case of *P. aeruginosa* infection, inflammasome activation increases the severity of the disease. However, these experiments were only performed using T3SS⁺ isolates.

This manuscript presents the study of the effect of inflammasome activation upon mice infection with an ExlA⁺ strain, using animals lacking caspase1, essential for the pro-inflammatory response normally induced by inflammasome.

In this project, I was mainly involved in the purification of neutrophils from the mice bone-marrow. I assessed the phagocytosis capacity of PMNs from the different animals. I also quantified IL-18 from BALs of infected mice.

RESEARCH ARTICLE

Inflammasome activation by *Pseudomonas aeruginosa*'s ExlA pore-forming toxin is detrimental for the host

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Abstract

During acute *Pseudomonas aeruginosa* infection, the inflammatory response is essential for bacterial clearance. Neutrophil recruitment can be initiated following the assembly of an inflammasome within sentinel macrophages, leading to activation of caspase-1, which in turn triggers macrophage pyroptosis and IL-1 β /IL-18 maturation. Inflammasome formation can be induced by a number of bacterial determinants, including Type III secretion systems (T3SSs) or pore-forming toxins, or, alternatively, by lipopolysaccharide (LPS) via caspase-11 activation. Surprisingly, previous studies indicated that a T3SS-induced inflammasome increased pathogenicity in mouse models of *P. aeruginosa* infection. Here, we investigated the immune reaction of mice infected with a T3SS-negative *P. aeruginosa* strain (IHMA879472). Virulence of this strain relies on ExlA, a secreted pore-forming toxin. IHMA879472 promoted massive neutrophil infiltration in infected lungs, owing to efficient priming of toll-like receptors, and thus enhanced the expression of inflammatory proteins including pro-IL-1 β and TNF- α . However, mature-IL-1 β and IL-18 were undetectable in wild-type mice, suggesting that ExlA failed to effectively activate caspase-1. Nevertheless, caspase-1/11 deficiency improved survival following infection with IHMA879472, as previously described for T3SS+ bacteria. We conclude that the detrimental effect associated with the ExlA-induced inflammasome is probably not due to hyperinflammation, rather it stems from another inflammasome-dependent process.

KEYWORDS

microbial-cell interaction, pseudomonads, toxins, virulence

1 | INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which exploits several virulence factors to achieve infection of various organs (Gellatly & Hancock, 2013; Hauser, 2009; Williams, Dehnbostel, & Blackwell, 2010). One of its most potent virulence factors is its type 3 secretion system (T3SS) through which it delivers several effectors (ExoS, ExoT, ExoY and ExoU) into the cytoplasm of target cells. Most strains also display a flagellum, type 4 pili, and LPS at their surface, collectively called pathogen-associated molecular patterns (PAMPs)

These PAMPs can trigger the innate immune system (Hayward, Mathur, Ngo, & Man, 2018) through binding to toll-like receptors (TLRs) located at the surface of sentinel macrophages or dendritic cells. TLR triggering induces the synthesis of a large number of proteins, including inflammatory cytokines such as the IL-1 β precursor (pro-IL-1 β), and components of the inflammasome complex (see below).

In addition, the T3SS machinery per se, by forming a pore in the macrophage plasma membrane, can activate an intracellular sensor of innate immunity, the NOD-like receptor (NLR) family CARD domain

containing 4 (NLRC4) (Hayward et al., 2018; Man & Kanneganti, 2016). The NLRC4-associated inflammasome is a multiprotein complex which activates IL-1 β and IL-18 by inducing their cleavage by caspase-1, and their release into the extracellular milieu following plasma membrane rupture, as a result of a cell death process called pyroptosis. Noncanonical inflammasome activation has also been described for Gram-negative bacteria. This process involves caspase-11, which can directly trigger pyroptosis and indirectly induce IL-1 β /IL-18 cleavage through activation of the NLR family pyrin domain containing 3 (NLRP3)-associated inflammasome. The resulting IL-1 β (and to a lesser extent IL-18) secretion contributes significantly to enhancing the inflammatory response during its initial phases. The immune reaction is subsequently amplified by several other pro-inflammatory cytokines and chemokines secreted by immune and non-immune cells, a process referred to as a 'cytokine storm', which leads to recruitment of circulating neutrophils to the infected organ (Garlanda, Dinarello, & Mantovani, 2013; Potey, Rossi, Lucas, & Dorward, 2019).

In general, infection with *P. aeruginosa* strains leads to a massive recruitment of neutrophils. In *P. aeruginosa*-induced pneumonia, a high number of neutrophils penetrates the alveoli and accumulates in the bronchi, where they actively combat invading bacteria. However, *P. aeruginosa* is generally well adapted to hyperinflamed environments and can thrive in this bacteriocidal milieu by adapting its metabolism. The mechanisms deployed include producing a protective biofilm and injecting T3SS effectors into neutrophils. These effectors can block phagocytosis, inhibit reactive oxygen species (ROS) production, and induce apoptosis (ExoS and ExoT) (Rangel, Logan, & Hauser, 2014; Sun, Karmakar, Taylor, Rietsch, & Pearlman, 2012; Vareechon, Zmina, Karmakar, Pearlman, & Rietsch, 2017) or necrosis (ExoU) (Diaz et al., 2008).

Although the inflammatory reaction remains the main weapon against *P. aeruginosa* in acute infections, several groups have demonstrated that it may also be deleterious for the host (Cohen & Prince, 2013; Faure et al., 2014; Schultz et al., 2002; Thakur, Barrett, Hobden, & Hazlett, 2004; Thakur, Barrett, McClellan, & Hazlett, 2004; Veliz Rodriguez et al., 2012). By using mice deficient in various inflammasome proteins, including caspase-1 and caspase-11, inhibitors of IL-1 β , or genetically deficient mice for components of the IL-1 β pathway, these groups showed that inactivating the inflammasome or preventing IL-1 β action diminished the severity of disease in infected mice. However, contradictory data have been published in the literature regarding the role of the inflammasome in bacterial clearance, possibly owing to the genetic and phenotypic diversity of *P. aeruginosa* strains (Lin & Kazmierczak, 2017).

In recent years, our group and others have identified phylogenetic outliers of *P. aeruginosa* phylum, that were named PA7-like strains, in reference to the first fully sequenced isolate of the group, PA7 (Roy et al., 2010). PA7-like strains have now been isolated worldwide from patients with acute or chronic infections affecting a range of organs (Freschi et al., 2018; Reboud et al., 2016). Several features distinguish PA7-like strains from 'classical' *P. aeruginosa* strains, including their lack of the whole locus encoding the T3S machinery and genes for the

T3SS effectors (Elsen et al., 2014). The pathogenicity of these strains is mainly based upon a two-partner secretion system, ExlB–ExlA, which is only found in PA7-like strains. ExlA (also called Exolysin) is a pore-forming toxin inducing necrosis in several mammalian cell types, and ExlB is the transporter located in the bacterial outer membrane that allows ExlA secretion into the extracellular medium (Basso, Ragno, et al., 2017; Reboud et al., 2016). Some of these strains are highly cytotoxic, a property that has been correlated with the amounts of ExlA secreted (Reboud et al., 2016). However, strain virulence in a mouse model of acute pneumonia was not directly linked to their toxicity towards cells nor to the amount of ExlA secreted (Reboud et al., 2016), suggesting that the immune reaction in response to the various PA7-like strains could differ.

Further work from our group revealed that the pore formed in the plasma membrane by ExlA can activate the NLRP3 inflammasome *in vitro*—whereas classical strains activate the NLRC4 inflammasome—leading to macrophage pyroptosis and IL-1 β secretion (Basso, Wallet, et al., 2017). Activation of the NLRP3 rather than the NLRC4 inflammasome may lead to different outcomes, as suggested by Iannitti et al. (2016). In this article, we studied the immune reaction induced in mice infected with an *exlA*+ strain that can trigger IL-1 β secretion from macrophages. More specifically, we evaluated the effect of inflammasome inactivation on mouse survival and the response to infection.

2 | RESULTS

2.1 | Inflammasome activation in macrophages by *exlA*+ strains

The capacity of bacteria to trigger inflammasome assembly and IL-1 β release by resident macrophages has a major impact on the host response to infection (Garlanda et al., 2013; Wonnenberg et al., 2016). We therefore investigated inflammasome induction following exposure to several *exlA*+ strains by analysing levels of pro (p)- and mature (m)-IL-1 β produced by infected macrophages. Mouse bone marrow-derived macrophages (BMDMs) were infected with 15 different *exlA*+ strains and the m-IL-1 β content of cellular lysates and supernatants was analysed at 3.5 hr post-infection (hpi) by Western blotting (Figure 1a). Pro-IL-1 β expression is induced by all *exlA*+ strains (except ATCC33359) at different levels, but they are all less efficient than the reference strain PAO1, an *exlA*– T3SS+ strain (ExoSTY+). In contrast, another *exlA*– T3SS+ (ExoUTY+) reference strain, PA14, was a poor inducer of p-IL-1 β , indicating that T3SS+ strains also differentially stimulate IL-1 β synthesis. m-IL-1 β was only detectable when cells were infected with IHMA567230, IHMA879472, or BLO43, however the signals were much less intense than observed following infection with PAO1. The profiles of IL-1 β contents in supernatants were confirmed by ELISA (Figure 1b). It should be noted that the small discrepancies between the Western blot and the ELISA data are likely due to the fact that the ELISA technique does not distinguish between the different forms of IL-1 β .

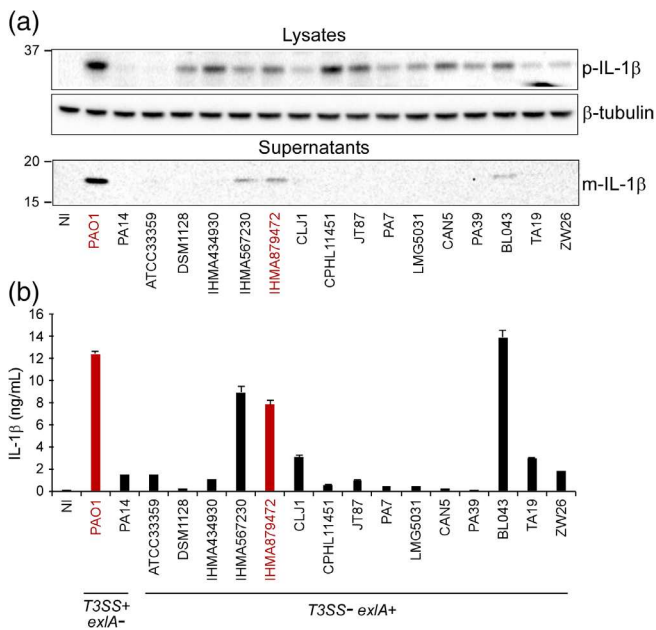


FIGURE 1 IL-1 β secretion in culture supernatants from infected BMDM. BMDMs were incubated with 15 T3SS- *exIA*+ strains, or T3SS+ *exIA*- strains (PAO1 and PA14) at MOI 10, or were left NI. Culture media and cell lysates were collected at 3.5 hpi. (a) Both the lysates (above) and the supernatants (below) were assayed for IL-1 β (either pro: p or mature: m) by Western blot. β -tubulin was used as loading control for the lysates. (b) IL-1 β concentrations in the supernatants assayed by ELISA. This analysis was performed twice. Strains used in subsequent studies are indicated in red. BMDM, bone marrow-derived macrophage; NI, non-infected

Altogether, there is no correlation between priming (induction of expression) and secretion of the mature form of IL-1 β . In conclusion, secretion of the active form of IL-1 β is more related to the capacity of *exIA*+ strains to activate the inflammasome than to induce its expression.

IL-18 is another interleukin processed by the inflammasome and secreted after macrophage pyroptosis (Hayward et al., 2018; Man & Kanneganti, 2016). IL-18 was not detected by Western blot in any supernatants of BMDMs infected by *exIA*+ strains (data not shown), suggesting that IL-18 is not involved in the immune response to these types of strains; a result that was confirmed *in vivo* (see below).

To combine virulence and m-IL-1 β response, we selected strain IHMA879472 (hereafter IHMA) for further investigation.

2.2 | Inflammasome activation is detrimental for mice infected with the IHMA strain

As mentioned above, several reports indicated that in mouse pneumonia models involving T3SS+ strains (either ExoS+ or ExoU+), survival was enhanced by inhibiting inflammasome induction (Cohen & Prince, 2013; Faure et al., 2014; Schultz et al., 2002; Thakur, Barrett, Hobden, & Hazlett, 2004; Thakur, Barrett, McClellan, & Hazlett, 2004; Veliz Rodriguez et al., 2012). To examine the impact of inflammasome

induction by IHMA (*exIA*+), we infected WT and caspase-1/caspase-11 KO (hereafter KO) mice by inhalation of a bacterial suspension. The KO mice used for this study induce neither conventional nor unconventional activation of IL-1 β , and cannot trigger pyroptosis (Basso, Wallet, et al., 2017). As with T3SS+ strains, mice lacking *caspase-1/caspase-11* genes survived significantly better than WT animals following infection with IHMA (Figure 2a), indicating that NLRP3-inflammasome activation is also detrimental in mice infected with an *exIA*+ strain. Mice (either WT or KO) infected with IHMA Δ *exIA* (IHMA Δ) strain all survived, indicating that ExIA is the major virulence factor deployed by IHMA *in vivo*.

Caspase-1 expression in WT lungs was strongly induced by IHMA and moderately by IHMA Δ (Figure 2b). This difference suggests that more inflammatory cells are recruited in lungs infected by IHMA than by IHMA Δ .

To directly assess the effects of IHMA on lung histology, infected mice were euthanized at 18 hpi and lung sections analysed by transmission electron microscopy (Figure 2c). Frequent interruptions of the pneumocytic lining of the alveoli (between arrowheads in Figure 2c) was observed in all mice (both WT and KO), confirming the cytolytic effect of IHMA (Basso, Wallet, et al., 2017). In contrast, no pneumocyte alteration was detected in IHMA Δ -infected lungs (WT or KO), confirming that pneumocyte disruption was caused by ExIA.

As disruption of the pneumocyte layer can affect permeability of the alveolo-capillary barrier, and to determine whether inflammasome inactivation affects this parameter, we measured the protein content of broncho-alveolar lavage (BAL) fluids (Figure 2d). Consistent with the histological data, IHMA-infected lungs were more permeable than those infected with IHMA Δ . Inactivation of *caspase-1/caspase-11* had no impact on permeability, indicating that the effect is a toxin-only effect. PAO1-infected mice showed a lower effect of this strain on barrier permeability, even though PAO1 is more virulent than IHMA *in vivo* (Reboud et al., 2016).

Pseudomonas aeruginosa exIA+ strains can potentially induce necrosis of different cell types in the lung: epithelial cells, when ExIA is secreted, macrophages, when pyroptosis is activated, and neutrophils, when neutrophil extracellular traps are produced by a process called NETosis (Delgado-Rizo et al., 2017). To further examine cell necrosis induced in infected lungs, BAL fluids were assessed to determine the β -actin (Figure 2e) and lactate dehydrogenase (LDH) (Figure 2f) levels. These two intracellular proteins are released after plasma membrane rupture. IHMA, and to a lower extent IHMA Δ , caused a significant increase in LDH activity. The difference in LDH activity between mice infected with IHMA and IHMA Δ strains was consistent with the degree of disruption of the pneumocytic lining observed in Figure 2c. The fact that IHMA Δ also induced some necrosis (Figure 2f) indicates that bacterial factors other than ExIA can trigger cell death, possibly by promoting neutrophil NETosis (see the confirmation below on isolated neutrophils), rather than macrophage pyroptosis, that requires ExIA activation of the inflammasome. Thus, our data suggest that IHMA is capable to induce both pneumocyte and neutrophil necrosis in the lung. PAO1 also induced necrosis in the

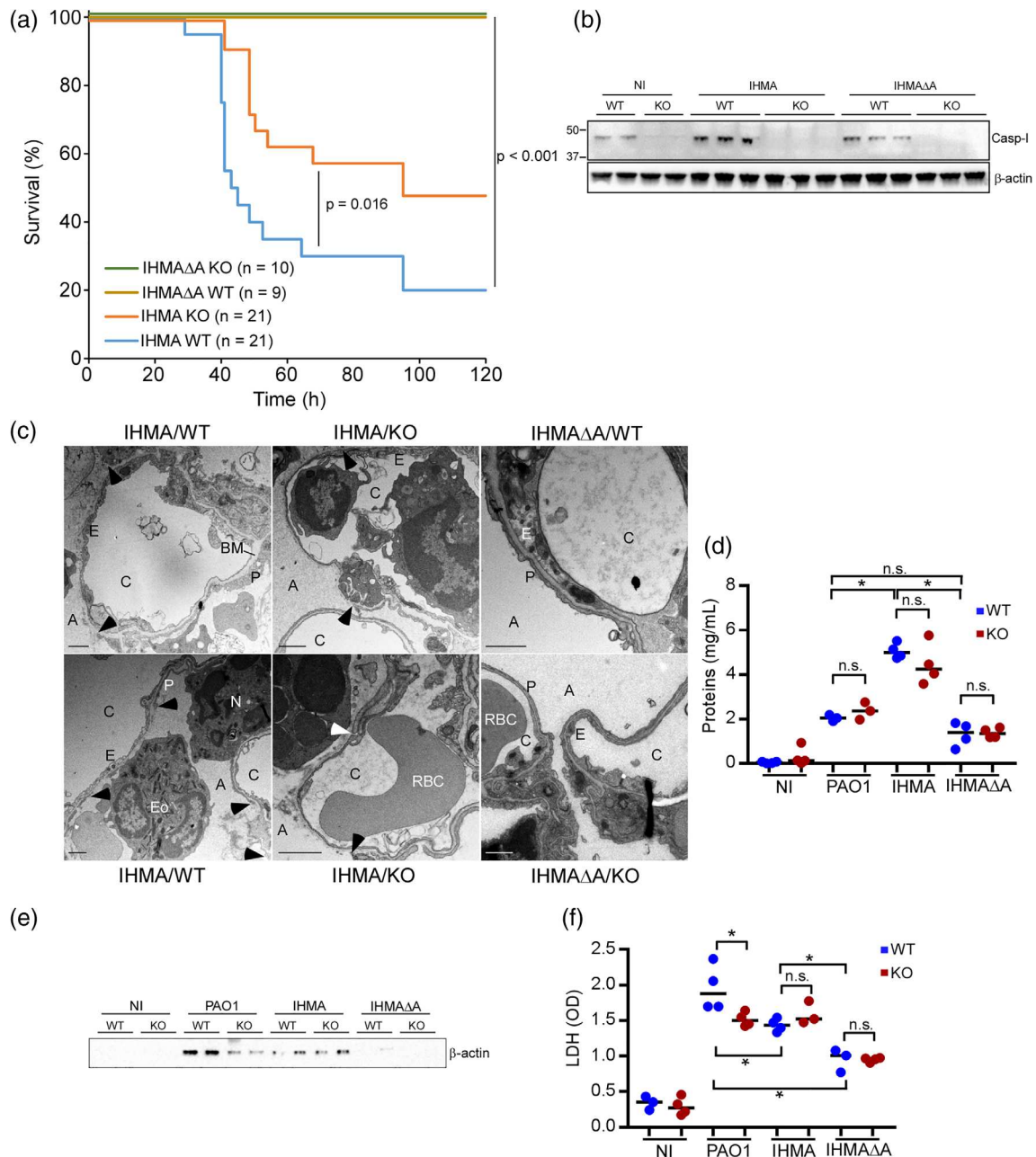


FIGURE 2 Infection of wild-type or caspase-1/caspase-11-deficient mice by IHMA or IHMA Δ A. (a) Mice, either WT or caspase-1/caspase-11 deficient (KO), were infected intranasally with IHMA or IHMA Δ A strains (10^7 bacteria), and their survival was monitored for 5 days. The number of mice per condition is indicated. Significance of differences between survival rates for WT and KO mice infected with IHMA, as well as differences in survival rates for WT mice infected with IHMA or IHMA Δ ex1A, were determined by the Log-Rank method, and the probability is shown on the graph. (b) Lung extracts were produced at 18 hpi and analysed by Western blotting using a caspase-1 antibody. β -actin was used as loading control. (c) Transmission electron micrographs of lung sections from IHMA- or IHMA Δ A-infected mice (WT and KO) euthanized at 18 hpi. Two representative images are shown for IHMA/WT and IHMA/KO and one for IHMA Δ A/WT and IHMA Δ A/KO. Lack of pneumocytic coverage along alveoli is shown between arrowheads. A, alveolus; BM, basement membrane; C, capillary; E, endothelium; Eo, eosinophil; N, neutrophil; P, pneumocyte; RBC, red blood cell. (d) BALs were collected post-mortem from mice euthanized at 18 hpi ($n = 4$ per condition). Protein concentrations were measured and plotted; median values are shown (bars). Differences between WT and KO were not significant according to an unpaired t test. Differences between bacterial strains in WT mice were significant according to an ANOVA test; multiple comparisons were performed using a Tukey test, and the results are indicated by (*) or n.s. Differences were all significant compared to the NI condition (not shown). (e) β -actin contents in BALs was analysed by Western blot. (f) LDH activities were measured in BALs and plotted along with median values. Statistical analyses were performed as in (d). BAL, broncho-alveolar lavage; LDH, lactate dehydrogenase

lungs, partially due to pyroptosis, as both markers were higher in BAL fluids from WT than from KO animals.

In summary, taken together, our results indicate that IHMA has a major effect on pneumocyte and probably neutrophil cell death, whereas PAO1 mainly induces macrophage lysis.

2.3 | Bacterial growth and dissemination

Bacterial loads in lungs, liver and spleen were determined at 18 hpi to assess the role played by caspase-1/caspase-11 in the capacity of IHMA to proliferate in the lungs and to disseminate to distant organs (Figure 3). The bacterial amounts found in IHMA-infected lungs at 18 hpi were similar to the initial load (10^7), suggesting that IHMA did not thrive in lungs over this time period. In sharp contrast, the population of PAO1 (Figure 3) dramatically increased over the same time period. Conversely, the IHMA $\Delta\Delta$ bacterial load decreased, indicating that ExIA is required for sustained bacterial population of this organ, probably as it plays a role in IHMA's mechanisms of defence against the immune system. IHMA and PAO1 survived better in the presence of caspase-1/caspase-11 than in their absence, a feature previously observed for the T3SS⁺ strain PAK (Cohen & Prince, 2013).

In conclusion, IHMA appears to be less resistant than PAO1 to the immune system, probably because there is no equivalent of the T3SS effectors capable of preventing neutrophil bacteriocidal action.

As for PAO1, IHMA disseminated to the liver and spleen, whereas IHMA $\Delta\Delta$ remained confined to the lungs, confirming that ExIA is key for bacterial spread in the body (Bouillot et al., 2017), like the T3SS for classical strains (Allewelt, Coleman, Grout, Priebe, & Pier, 2000; Kudoh, Wiener-Kronish, Hashimoto, Pittet, & Frank, 1994; Rangel, Diaz, Knoten, Zhang, & Hauser, 2015; Shaver & Hauser, 2004; Vance, Rietsch, & Mekalanos, 2005).

2.4 | Mature-IL-1 β is undetectable in IHMA-infected lungs, but IL-1 α is highly produced

To assess IHMA's capacity to promote IL-1 β synthesis *in vivo*, we first used ELISA to measure the amounts of total IL-1 β (pro and mature) in

lung extracts (Figure 4a). For both IHMA- and IHMA $\Delta\Delta$ -infected lungs, there was a small non-significant increase in IL-1 β at 8 hpi; no difference was observed between WT and KO lungs. In contrast, at this time point, PAO1 had already induced a significant IL-1 β response in WT mice, but not in KO mice. This difference between mouse strains continued to 18 hpi. At this later time point, IL-1 β levels were also significantly increased in lungs infected with IHMA, and to a lower extent in those infected with IHMA $\Delta\Delta$, but again no difference between WT and KO mice was observed with these bacterial strains (Figure 4a). As only mature-IL-1 β produced upon caspase-1-dependent cleavage is active (Afonina, Muller, Martin, & Beyaert, 2015), we analysed lung extracts (18 hpi) by Western blot to discriminate between the two forms of IL-1 β (Figure 4b). Mature-IL-1 β was only detected in PAO1-infected WT mice, whereas strong pro-IL-1 β signals were detectable in all infected conditions. Taken together, we conclude that both IHMA and IHMA $\Delta\Delta$ can promote pro-IL-1 β expression via TLR activation, although not as early or as strongly as PAO1. The slight difference observed between IHMA- and IHMA $\Delta\Delta$ -infected lungs in the ELISA results suggests that ExIA contributes to the induction of pro-IL-1 β expression. However, no mature-IL-1 β was detected in IHMA-infected lungs, suggesting that ExIA is a poor inflammasome activator, or that NLRP3 is less efficient than NLRC4 when it comes to activating caspase-1. It should be noted that electrophoretic gels had to be overloaded to detect m-IL-1 β by Western blot in PAO1-infected lungs, indicating that only very small amounts of the mature form were present even for this strain, as previously reported (Galle et al., 2008). Thus, most of the pro-IL-1 β synthesized remained unused, and only minute amounts of active IL-1 β were detected following infection with PAO1, and any active IL-1 β produced following IHMA infection was at undetectable levels. However, extremely low amounts of IL-1 β is nevertheless sufficient to induce inflammatory reactions, as previously noted (Garlanda et al., 2013).

As for infected BMDM supernatants, IL-18 was at background levels in lung extracts when mice were infected with IHMA or IHMA $\Delta\Delta$ (Figure 4c), confirming that IL-18 does not play a role in the immune response to IHMA.

IL-1 α , an interleukin targeting the same receptor as IL-1 β (and thus triggering the same effects), but that does not require inflammasome

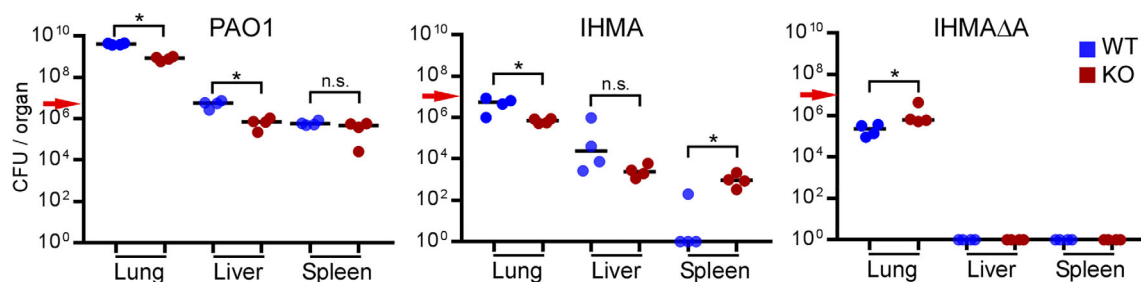


FIGURE 3 Bacterial growth in lungs and dissemination in liver and spleen. Mice infected with PAO1, IHMA or IHMA $\Delta\Delta$ were euthanized at 18 hpi, and their lungs, liver and spleen were homogenised. Serial dilutions of the homogenates were plated on LB plates for CFU counting. Data are shown with medians (bars). Statistical differences between WT and KO mice were calculated using a Mann-Whitney test. The red arrows indicate the initial bacterial load

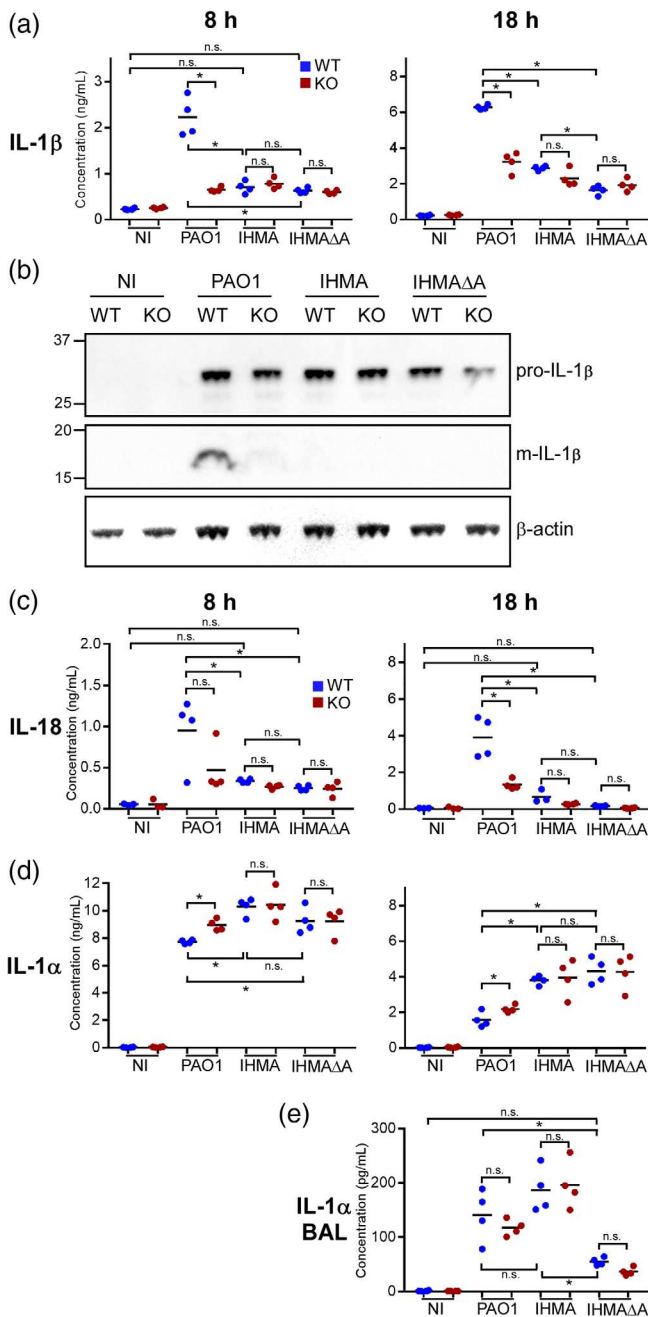


FIGURE 4 IL-1 β , IL-18 and IL-1 α production in infected lungs. (a) IL-1 β concentration was measured by ELISA in lung extracts at 8 and 18 hpi. Results were plotted together with mean values (bars). (b) Lung extracts at 18 hpi were also analysed by Western blotting, using IL-1 β antibody and β -actin as control. Two gels were used: one for pro-IL-1 β / β -actin, and one for mature-IL-1 β . To detect mature-IL-1 β , gels were overloaded with protein, resulting in a distorted migration profile. (c) IL-18 concentration was measured by a bead-based immunoassay in lung extracts at 8 and 18 hpi. (d) IL-1 α concentration was measured by ELISA in lung extracts at 8 and 18 hpi. (e) IL-1 α concentration was measured by ELISA in BAL supernatants at 18 hpi. All statistical analyses (*t* test and ANOVA) were performed as described for Figure 2

activation, is rapidly and massively upregulated by IHMA and IHMA $\Delta\Delta$ strains, independent of the presence of caspase-1/caspase-11 as

expected (Figure 4d). IL-1 α was also detected in BAL supernatants from IHMA-infected mice (Figure 4e), showing that IL-1 α is indeed secreted and thus can interact with the IL-1 receptor (IL-1R). Likewise, IL-1 α production may compensate for the lack of substantial secretion of active IL-1 β and may trigger the production of secondary inflammatory cytokines, like IL-6, IL-17a and KC. Interestingly, IL-1 α contents of BAL supernatants from IHMA $\Delta\Delta$ -infected mice were significantly lower, suggesting that ExIA-dependent cell lysis was required for IL-1 α release.

2.5 | Induction of pro-inflammatory cytokines by IHMA

Several pro-inflammatory cytokines (IL-6, IL-17a and TNF- α) and a chemokine (KC) were significantly induced in IHMA-infected lungs. Levels of all inflammatory mediators except TNF- α were increased between 8 and 18 hpi.

The most striking increase was observed for IL-6, a cytokine involved in neutrophil maturation and recruitment (Figure 5). IL-6 levels were similar in PAO1- and IHMA-infected lungs, but were strikingly lower at 18 hpi in the absence of caspase-1/caspase-11. Furthermore, IHMA $\Delta\Delta$ failed to induce IL-6 expression in infected lungs at 18 hpi. Thus, ExIA is required for IL-6 expression, and both caspase-1/caspase-11-dependent and -independent pathways are involved.

Two other mediators of neutrophil recruitment—IL-17a and especially KC—were also induced by IHMA at 18 hpi, but at lower levels than with PAO1. Similar to our result for IL-6, caspase-1/caspase-11 deficiency was associated with lower IL-17a and KC levels, and their expression was negligible in IHMA $\Delta\Delta$ -infected lungs. Thus, in addition to ExIA, to enhance IL-6, IL-17a, and KC expression, the inflammasome must be activated. This conclusion is consistent with previous reports using other strains (Gasse et al., 2011; Rosales, 2018; Schultz et al., 2002; Slaats, Ten Oever, van de Veerdonk, & Netea, 2016).

TNF- α is another chemo-attractant for neutrophils that also facilitates their extravasation by acting on endothelial cells. TNF- α expression was found to be induced to similar levels in all infected conditions, regardless of strain type or mouse genetic background.

Taken together, our results show that IHMA-infected WT mice can mount an inflammatory cytokine response, which is mostly dependent on ExIA and partly on caspase-1/caspase-11 activation. As mentioned previously, massive IL-1 α production in IHMA-infected lungs is likely to provide the alarming signal triggering pro-inflammatory cytokine synthesis in the infected lungs.

Interestingly, the anti-inflammatory cytokine IL-10 was detected at 8 and 18 hpi in IHMA $\Delta\Delta$ -infected lungs, but not in IHMA-infected lungs. This result suggests that mice infected with the avirulent IHMA $\Delta\Delta$ strain were at a very early stage in the resolution phase of inflammation, a process characterised by IL-10 production. A similar increase in IL-10 levels was observed in PAO1-infected lungs at 18 hpi. The main difference between the two cases was that the increase was caspase-1/caspase-11-independent for IHMA $\Delta\Delta$ and caspase-1/caspase-11-dependent for PAO1, suggesting different mechanisms of activation.

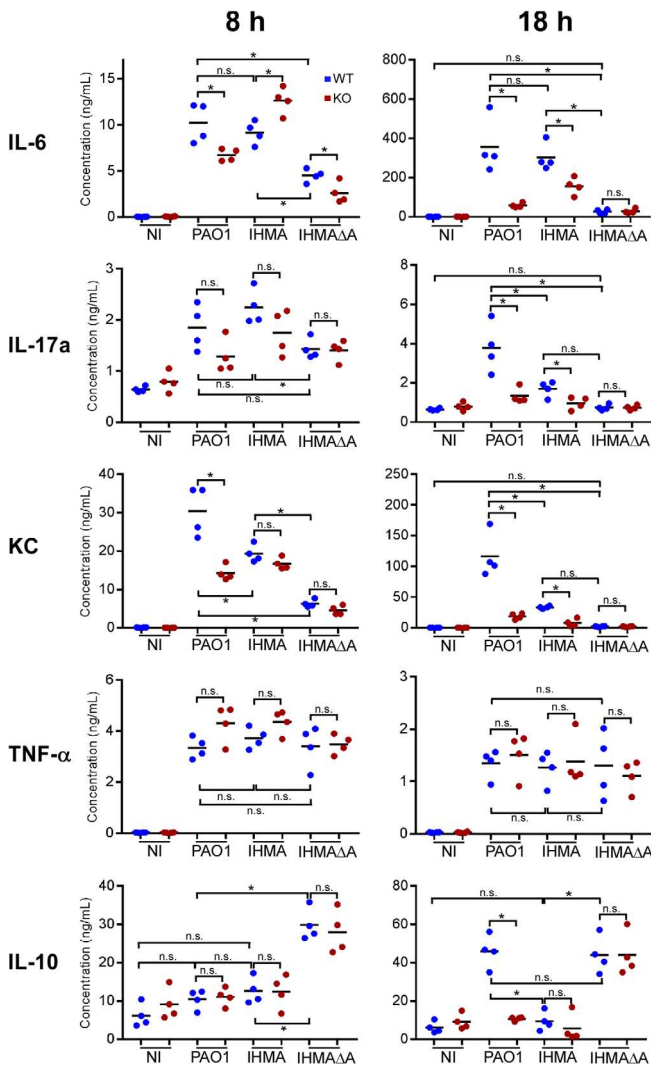


FIGURE 5 Additional inflammatory cytokines and chemokine in infected lungs. Levels of IL-6, IL-17a, KC, TNF- α and IL-10 in lung extracts at 8 and 18 hpi were measured by ELISA and plotted alongside mean values. Significance of differences between WT and KO data were calculated using an unpaired *t* test, or Mann-Whitney test when distribution was not normal (i.e. for IL-17a at 18 hpi, TNF- α at 18 hpi, IL-10 at 18 hpi). For all cytokines, differences between bacterial strains in WT mice were significant according to a one-way ANOVA test; multiple comparisons were performed using a Tukey's test and the results are indicated with a (*) or n.s. Differences were all significant relative to NI, unless indicated with n.s.

A diagram summarising our results related to cytokine production is presented in Figure 6.

2.6 | Blocking IL-1 receptor has no impact on IHMA-infected mouse survival

The differences in mouse survival observed in Figure 2a between WT and Caspase-1/caspase-11 KO mice infected by IHMA may be caused

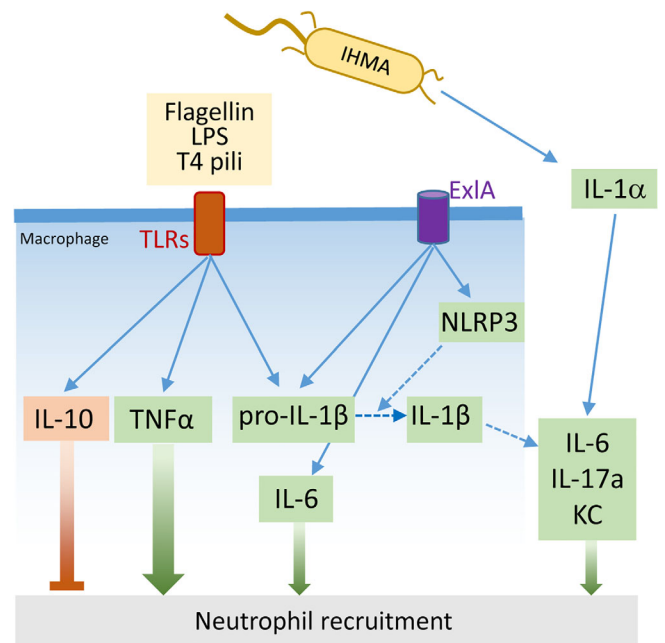


FIGURE 6 Proposed mechanisms of inflammatory cytokine activation by IHMA. The diagram summarises the main results from Figures 4 and 5. IHMA can activate TLR receptors at the surface of macrophages, via the release of flagellin, pili and LPS, in an ExlA-independent manner. TLR activation results in IL-10, TNF- α and pro-IL-1 β expression. ExlA has a strong and direct effect on IL-6 production, but only a small but significant impact on pro-IL-1 β expression. Production of mature IL-1 β by ExlA-induced NLRP3 inflammasome was not detected in vivo; the dotted lines indicate that IL-1 β activation was only observed in vitro. Conversely, IL-1 α expression was strongly upregulated by IHMA, which is likely responsible for enhancement of IL-6, IL-17a and KC synthesis

by the hyperinflammation-induced by pro-inflammatory cytokine secretion, as suggested by previous works (Cohen & Prince, 2013; Faure et al., 2014; Schultz et al., 2002; Thakur, Barrett, Hobden, & Hazlett, 2004; Thakur, Barrett, McClellan, & Hazlett, 2004; Veliz Rodriguez et al., 2012), or by the activation of the inflammasome itself, as recently reported (Wu et al., 2019). To discriminate between these two hypotheses, we used a pharmacological inhibitor of IL-1R, anakinra (Cavalli & Dinarello, 2018), in mouse infection assays. Mice were administered with anakinra (10 mg/kg), as previously reported (Norelli et al., 2018), 1 hr before infection, and then daily. We first examined anakinra efficiency on IL-6 production at 18 hpi (Figure 7a). Anakinra administration dramatically reduced IL-6 content of infected lungs compared to untreated mice. Next, we performed survival experiments on mice treated with anakinra or untreated (Figure 7b). The two survival curves were not significantly different. Hence, IL-1R blocking had no impact on mouse survival after IHMA infection. As IL-18 is not produced following IHMA infection (Figure 4c), these results suggest that the decrease in mouse death observed in absence of caspase-1/caspase-11 is not due to the lack of IL-1 β /IL-18 secretion, but to the absence of a functional inflammasome, per se.

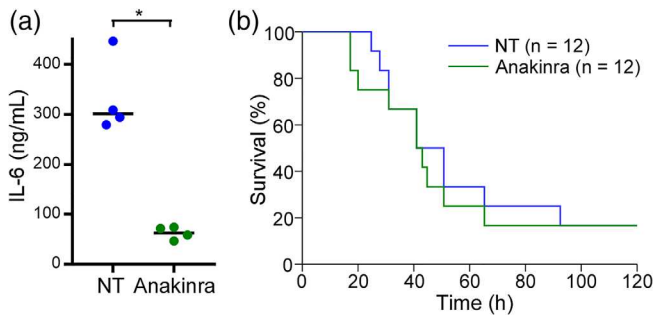


FIGURE 7 Effect of IL-1 receptor inhibition on mouse survival. (a) Mice were either treated with the IL-1 receptor inhibitor anakinra at 10 mg/kg or untreated ($n = 4$ in each condition), and infected by IHMA 1 hr later. Mice were euthanized at 18 hpi, IL-6 concentrations were measured in lung extracts and represented together with the median. Statistical differences were calculated with Student's t test; $*p < .0001$. (b) Mice were either untreated or treated with anakinra (10 mg/kg 1 hr before infection and then daily), and infected with IHMA. Shown is the Kaplan–Meyer representation of the survival curves in each condition. No statistical difference was obtained with the Log-Rank test

2.7 | Neutrophil recruitment and activity in IHMA-infected lungs

Having shown that IHMA-induced a pro-inflammatory cytokine response, we next monitored the presence of neutrophils in infected lungs by assaying myeloperoxidase (MPO) activity in lung extracts (Figure 8a). MPO activity was increased to a similar extent in all infected WT mice as early as 8 hpi. Caspase-1/caspase-11 deficiency significantly altered neutrophil recruitment in IHMA- and PAO1-infected lungs. At 18 hpi, MPO activity was increased approximately 10-fold over baseline in all infected conditions. For IHMA, neutrophil infiltration was increased due to the presence of both ExIA and caspase-1/caspase-11. PAO1 yielded a significantly higher response than IHMA, but the increase was not significantly different in animals lacking caspase-1/caspase-11.

From these results, we conclude that IHMA can trigger neutrophil recruitment almost in the same range as PAO1, and although the presence of ExIA and caspase-1/caspase-11 both contribute to this process, they are not absolutely required, as high MPO activities were also detected in IHMA $\Delta\Delta$ -infected KO mice.

To examine any association between bacteria and neutrophils in infected lungs, we analysed lung histological sections labelled with anti-LPS antibody (for bacteria), and with Hoechst to identify neutrophils thanks to their characteristic nuclear shape. In infected lungs, neutrophils infiltrated the alveoli and accumulated in the bronchi, where they were associated with IHMA or IHMA $\Delta\Delta$ bacteria (Figure 8b), thus suggesting that neutrophils were actively internalising bacteria.

To characterise the phagocytic capacity of neutrophils in both mouse strains, neutrophils were isolated from WT and KO mice and were incubated with pHrodo Green *Escherichia coli* (dead *E. coli* labelled with a dye which fluoresces green upon phagosome

acidification). Cells exposed to these bioparticles were then analysed by flow cytometry. Similar percentages of fluorescence intensity were recorded for both WT and KO neutrophils, indicating that they were equally capable of internalising and processing bacteria (Figure 8c).

Pseudomonas aeruginosa's T3SS effectors have been reported to induce neutrophil apoptosis, pyroptosis or necrosis (Diaz et al., 2008; Diaz & Hauser, 2010; Rangel et al., 2014; Sun et al., 2012; Vareechon et al., 2017). To investigate the capacity of IHMA to induce neutrophil death, the survival of infected neutrophils was determined in kinetic experiments. The numbers of remaining neutrophils were counted by microscopy at several time points (Figure 8d). The area under the curve (AUC) was plotted for each strain (PAO1, IHMA and IHMA $\Delta\Delta$) for neutrophils from WT or KO mice (Figure 8e). Incubation with IHMA and IHMA $\Delta\Delta$ induced similar death patterns, with significantly higher mortality than recorded in non-infected conditions and significantly lower mortality than following exposure to PAO1. No difference between WT and KO neutrophils was detectable in any condition, except with PAO1, to which KO neutrophils were slightly less sensitive than WT neutrophils. This feature suggests that, unlike IHMA, PAO1 can induce a low level of pyroptosis in this cell type, as previously shown (Ryu et al., 2017). Hence, IHMA-induced a cell death program in neutrophils that is independent of ExIA and does not result from pyroptosis. This program could partly account for the release of cytoplasmic proteins detected in BAL fluids (Figure 2d,e).

In addition to their phagocytic capacity, neutrophils can immobilise and kill bacteria by forming neutrophil extracellular traps (NETs), composed of chromatin and proteins from secretory vesicles (Delgado-Rizo et al., 2017; Rosales, 2018). *P. aeruginosa* has been shown to induce NET formation in various models (Dwyer et al., 2014; Thanabalasuriar et al., 2019; Yoo et al., 2014; Young et al., 2011). To examine whether IHMA-induced neutrophil death was due to NET formation, infected neutrophils were labelled with Hoechst, to reveal their DNA, and with anti-histone H2AX antibodies, two markers of NETs (McDonald, Urrutia, Yipp, Jenne, & Kubes, 2012). As illustrated in Figure 8f, multiple figures showing networks containing DNA and H2AX, which are typical of NETs, were observed when neutrophils were infected with PAO1, IHMA or IHMA $\Delta\Delta$. Thus, IHMA can induce NET formation, thereby contributing to bacterial elimination.

3 | DISCUSSION

Inflammasome activation is a powerful system to rapidly trigger the inflammatory response in order to contain bacterial expansion. Experiments in mice with different genetic backgrounds showed that mouse strains where the immune response is delayed by only a few hours eliminate bacteria less efficiently and have a poorer outcome than mice mounting an earlier response (De Simone et al., 2014). Inflammasome induction is crucial to eradicate internal pathogens, such as *Listeria monocytogenes* (Sauer et al., 2011). However, uncontrolled inflammation may induce tissue damage and limit gas exchange in lungs, because of overwhelming neutrophil accumulation and

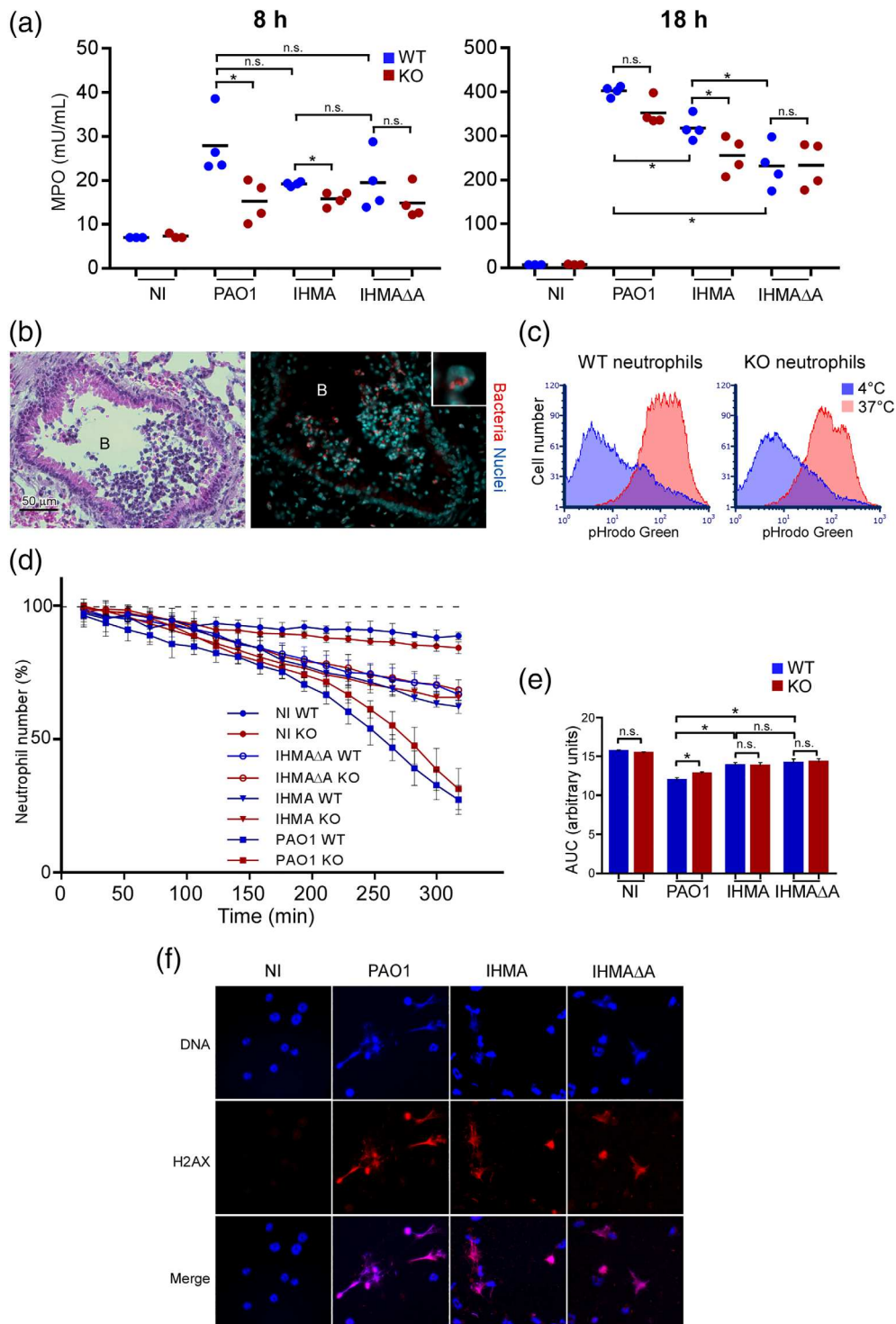


FIGURE 8 Neutrophil infiltration and activity in infected lungs. (a) Neutrophil content was monitored by measuring MPO activity in lung extracts collected at 8 and 18 hpi. Data are plotted with the corresponding mean value. Significance of differences between WT and KO data were calculated using the Mann–Whitney's test. Differences between bacterial strains in WT mice were significant according to a one-way ANOVA test; multiple comparisons were performed using a Tukey's test and the results are indicated with a (*) or n.s. Differences were all significant relative to NI. (b) Paraffin sections of IHMA-infected WT lungs were stained with haematoxylin–eosin to reveal morphological features (left), and with anti-LPS and Hoechst to reveal bacteria and nuclei, respectively (right). Images represent bronchi (b) containing neutrophils. The insert shows bacteria associated with a neutrophil at higher magnification. Similar data were obtained with KO mice and mice infected with IHMA $\Delta\Delta$ (not shown). (c) Phagocytosis assay using pHrodo Green *E. coli*. Neutrophils were incubated with the bioparticles for 1 hr, either at 4°C (inactive phagocytosis, used as control) or at 37°C (active phagocytosis) before analysis by flow cytometry. (d,e) Neutrophils from WT or KO mice were incubated in an automated microscope with IHMA, IHMA $\Delta\Delta$ or PAO1 at MOI 10, or were uninfected (NI). Phase-contrast images (nine per condition) were acquired every 15 min for 5 hr. Neutrophil numbers were counted using ImageJ and the means (\pm SD) were plotted as a function of time (d). (e) The areas under the curves (AUC) were calculated and are represented as means + SD. Statistical analyses were performed as described for Figure 2. (f) To visualise NETosis, infected cells were labelled with Hoechst for DNA (blue) and anti-H2AX antibodies (red). Intact neutrophils are only blue, and neutrophils subjected to NETosis are revealed by spread chromatin labelled by both markers. All experiments on neutrophils were repeated at least twice, with similar results. MPO, myeloperoxidase

excessive edema. In the mouse acute pneumonia model, inflammasome ablation increased host survival following infection with various T3SS+ *P. aeruginosa* strains (Cohen & Prince, 2013; Faure et al., 2014; Thakur, Barrett, Hobden, & Hazlett, 2004; Thakur, Barrett, McClellan, & Hazlett, 2004). This effect was attributed to the hyperinflamed status of the infected lungs when the inflammatory caspases are present. This hypothesis was recently challenged by Wu et al. (2019) who demonstrated that inflammasome activation triggers intravascular coagulation, resulting in host death. In their study, these authors showed that macrophage pyroptosis led to the formation of microvesicles decorated with Tissue Factor, which initiates the extrinsic coagulation cascade. This process was demonstrated to exist for both the T3SS-induced NLRC4 inflammasome, and non-canonical activation of the inflammasome by caspase-11, and provides a major insight into the surprising improvement in survival observed after inflammasome inactivation.

Pseudomonas aeruginosa strains are known to be highly heterogeneous, especially in terms of virulence factors and release of bacterial determinants (flagellin, pilin and LPS) stimulating inflammatory cytokine expression via TLR activation. The IHMA strain studied herein possesses pili and a flagellum (Reboud et al., 2016) and is indeed able to stimulate IL-1 β expression (Figures 1a and 4b).

Previous studies investigating the role of the inflammasome in host immune reaction to *P. aeruginosa*, used T3SS+ strains activating NLRC4 (Cohen & Prince, 2013; Faure et al., 2014; Iannitti et al., 2016; Schultz et al., 2002; Thakur, Barrett, Hobden, & Hazlett, 2004; Thakur, Barrett, McClellan, & Hazlett, 2004; Veliz Rodriguez et al., 2012). Here, we showed that inflammasome activation is also detrimental for mice infected with an *exlA*+ strain, although a different inflammasome is involved (NLRP3 vs. NLRC4) (Basso, Wallet, et al., 2017) and its inactivation has a moderate effect on inflammatory mediators. Indeed, IHMA-dependent inflammasome activation induced secretion of small amounts of m-IL-1 β in vitro and undetectable amounts in vivo (Figures 1 and 4). Furthermore, caspase ablation resulted in only a very small (albeit significant) relative decrease in neutrophil recruitment, as measured by MPO activity (Figure 8a). Finally, inhibition of IL-1R and the subsequent diminution of IL-6 synthesis did not modify mouse survival curve profile (Figure 7). Likewise, our data support the model proposed by Wu et al. (2019), rather than the hypothesis of hyperinflammation-induced host death.

The precise role of caspase-11 in the immune response to pathogens remains elusive. Caspase-11 is not involved in IL-1 β production when mice are infected with a T3SS+ strain (Cohen & Prince, 2013). We previously showed that *ExlA* failed to induce pyroptosis when macrophages lacked either NLRP3 or ASC (a central player of the NLRP3 inflammasome) (Basso, Wallet, et al., 2017). As caspase-11 can induce pyroptosis independently of NLRP3, our previous in vitro data excluded a role for caspase-11 in *ExlA*-induced pyroptosis. Therefore, it is probable that caspase-11 was not activated by *ExlA* in alveolar macrophages and did not contribute to NLRP3-dependent IL-1 β activation either. However, caspase-11 was shown to induce pneumocyte lysis (Wang et al., 2018). Thus, caspase-11 deficiency may also contribute to the improved mouse survival observed in

infected caspase-1/caspase-11 KO mice by limiting pneumocytic lesions.

As for the T3SS+ bacteria, the capacity of *exlA*+ strains to induce inflammasome assembly is highly variable and independent of its toxicity on non-immune cells. In a previous study, we showed that the hypervirulent strain CLJ1, which secretes high levels of *ExlA* but displays no flagellum nor type 4 pili, can grow in mouse lungs while remaining relatively non-immunogenic (Bouillot et al., 2017). In contrast, the IHMA bacterial population remained relatively stable in the lungs and dramatically increased pro-IL-1 β level, hence priming the expression of a major inflammasome substrate, as well as other inflammatory cytokines (including TNF- α and IL-10). As mentioned above, no m-IL-1 β was detectable in IHMA-infected lungs (Figure 4b). Nevertheless, IL-1 β is a very potent cytokine, acting at vanishingly low concentrations on cells (Garlanda et al., 2013). In addition, IL-1 α , interacting with the same receptor as IL-1 β , was massively produced in IHMA-infected lungs and was probably the main contributor of the enhancement of IL-6, IL-17a and KC production (Figure 5), thereby amplifying the inflammatory reaction. In addition to caspase-1/caspase-11, *ExlA* is absolutely required for IL-17a and KC synthesis, as they were not induced by IHMA $\Delta\Delta$ (Figure 5), indicating that the TLRs are not involved in the induction observed.

Furthermore, our data show that *ExlA* per se, possibly through pore formation, can stimulate pro-IL-1 β and IL-6 production (Figure 5). Two PFTs, staphylococcal Pantone-Valentine leukocidin and Listeriolysin O from *Listeria monocytogenes* (Kayal et al., 2002; Ma, Chang, Zhang, Zhou, & Yu, 2012), were shown to induce a stress response that can activate NF- κ B, thereby inducing an inflammatory response not relying on TLR activation. Hence, *ExlA* may use a similar pathway to stimulate pro-IL-1 β and IL-6 synthesis.

IL-10 is known to improve lung injury and survival in *P. aeruginosa* pneumonia (Chmiel et al., 1999; Guilbault et al., 2002; Sawa et al., 1997). The production of IL-10 in IHMA $\Delta\Delta$ -infected lungs, from 8 hpi, further indicates that the inflammation was promptly defused when mice were infected with IHMA $\Delta\Delta$, thereby preventing collateral damage to tissues. In contrast, IL-10 remained undetectable at 18 hpi in IHMA-infected lungs, suggesting that inflammation was still very active at this time point. In comparison, PAO1-infected lungs contained high IL-10 levels at 18 hpi, meaning that the inflammatory response was tempered at this stage. Thus, the pro-inflammatory phase may last longer with IHMA than with PAO1.

Neutrophils were massively recruited to IHMA-infected lungs, even in the absence of *ExlA* and the inflammatory caspases (Figure 8a). This result suggests that neutrophil recruitment mainly relies on secretion of TNF- α , or other untested cytokines, as levels of IL-6, IL-17a and KC remained low in the absence of both *ExlA* and the caspases. However, the presence of either the toxin or the caspases significantly increased lung MPO levels, in line with the known pro-inflammatory action of these cytokines.

IHMA and IHMA $\Delta\Delta$ bacteria were found associated with neutrophils in infected lung sections (Figure 8b). Part of the bacterial population was probably located inside neutrophils, as suggested by the insert image. Neutrophils from WT and KO mice were equally

competent for phagocytosis (Figure 8c), confirming previous data showing that inflammasome activation is not necessary for extracellular killing of bacteria, whereas it is required for the elimination of intracellular bacteria (Cohen & Prince, 2013). In addition, both IHMA and IHMA $\Delta\Delta$ induced NETosis of WT and KO neutrophils (Figure 8d–f), thus providing neutrophils with another opportunity to kill this bacterial strain. PAO1 induced a stronger NETosis response, although it thrives better in the lungs. This apparent discrepancy is consistent with a recent report showing that PAO1 can evade the antimicrobial activity of NETs by secreting a DNase (EddB) and a phosphatase (EddA) that both contribute to the bacteria's defence against killing (Wilton, Halverson, Charron-Mazenod, Parkins, & Lewenza, 2018). The genes encoding these two enzymes are also present in IHMA; further work will be required to clarify whether these enzymes are expressed in this strain and mount a similar defensive mechanism.

The pathogenic role of ExlA was confirmed in this study. As previously shown, ExlA is the major factor causing *exlA*+ strain virulence, as all IHMA $\Delta\Delta$ -infected mice survived (Figure 2a). Images of alveoli with partial pneumocytic lining were frequently observed when ExlA was present, but never seen in lungs infected with IHMA $\Delta\Delta$ or PAO1 (Figure 2c). Furthermore, IHMA $\Delta\Delta$ bacterial loads rapidly decreased in lungs, and bacteria did not spread to other organs in mice (Figure 3). ExlA is thus responsible for multiple pathogenic actions of IHMA. It is noteworthy that IHMA could be pathogenic and induced lethality without any notable increase in bacterial burden.

Similarly, IHMA can induce death in three different cell types in the infected lungs: (a) it induces ExlA-dependent necrosis of pneumocytes, which causes tissue lesions and increases pulmonary barrier permeability, (b) it can induce neutrophil death by the formation of NETs, and (c) it triggers macrophage pyroptosis, leading to increased host lethality by a process that is probably independent of an excessive inflammatory response (Figure 9).

This study also showed that *exlA*+ strains—when they activate the innate immune response—are poorly adapted to an inflammatory environment; these strains are less resistant to the action of

phagocytic cells, possibly because they have developed fewer counteracting mechanisms than T3SS+ strains.

In conclusion, whatever the pathway and the level of inflammasome activation by *P. aeruginosa*, activation of the inflammatory caspases is detrimental for the host. Therefore, targeting the inflammatory caspases may be beneficial for hosts infected by any strains of *P. aeruginosa*.

4 | EXPERIMENTAL PROCEDURES

4.1 | Ethics statement

All protocols in this study were conducted in strict compliance with the French guidelines for the care and use of laboratory animals. The protocols for mouse infection were approved by the institutional animal research committee (CETEA#44, project number 13-024 and 20-033) and the French Ministry for Research.

4.2 | *Pseudomonas aeruginosa* strains and culture

The strains used in this study (15 *exlA*+ strains and IHMA879472 $\Delta exlA$) are described elsewhere (Basso, Ragno, et al., 2017; Reboud et al., 2016). IHMA879472 genome sequence has been published recently (Kos et al., 2015). Reference strains PAO1 and PA14 were used for comparison. Bacteria were grown in liquid LB medium at 37°C with agitation until the cultures reached an optical density at 600 nm of 1.0.

4.3 | Production of BMDMs

BMDMs were prepared from the femurs and tibias of C57Bl/6 mice and differentiated as previously described (Basso, Wallet, et al., 2017).

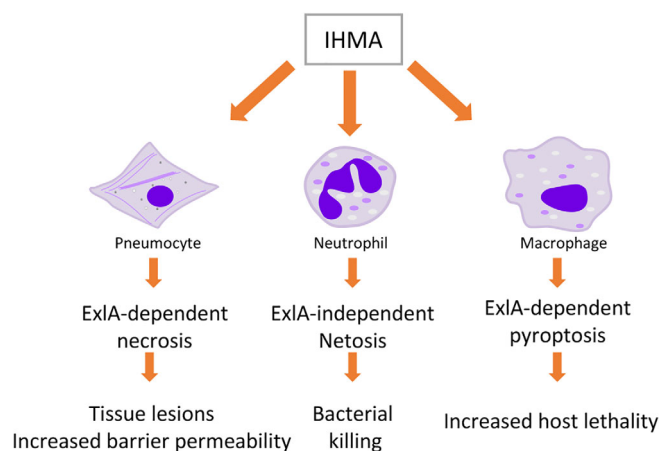


FIGURE 9 Action of *exlA*+ *P. aeruginosa* on pneumocytes, neutrophils and macrophages IHMA induces death of three cell types present in the lung. ExlA triggers pneumocyte necrosis, which induces the rupture of the alveolo-capillary barrier. IHMA promotes neutrophil NETosis, independent of ExlA action, as a response to bacterial invasion. ExlA promotes macrophage pyroptosis via NLRP3 activation

BMDMs were grown in bone marrow differentiation media: RPMI 1640 (Gibco) supplemented with 20% fetal bovine serum (Lonza), 2 mM glutamine (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco) and 10% L929-cell conditioned medium (LCCM) at 37°C, 5% CO₂ in non-tissue culture-treated dishes. Four days later, 10 mL of fresh differentiation medium was added and cultures were incubated for an additional 3 days. Twenty-four hours before exposure to bacteria, BMDMs were resuspended in BMDM culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 5% LCCM and 2 mM L-Glutamine).

4.4 | Analysis of IL-1β in infected BMDM lysates and supernatants

Cells were infected for 3.5 hr at MOI 10 with the bacterial strains. Then, supernatants were collected and cellular lysates were prepared. Both were analysed for their IL-1β contents by Western blotting using the anti-mouse IL-1β from R&D Systems (AB-401-NA). The β-actin antibody was used for loading control of the lysates. IL-1β concentrations in supernatants were measured by ELISA (BioLegend).

4.5 | Mouse pulmonary infection

Pathogen-free C57Bl/6 (WT) mice were obtained from Charles River Laboratories and C57Bl/6 *caspase-1/caspase-11* double knockout (KO) mice were kindly provided by Thomas Henry (Ciri, Lyon). Animals were housed and mated in the institute's animal care facility. Both males and females were used in equilibrated ratios. Bacteria from exponential growth (OD₆₀₀ = 1.0) were centrifuged and resuspended in sterile PBS at 1.67 × 10⁸ per mL for PAO1 and 3.33 × 10⁸ per mL for IHMA and IHMAΔA. Mice (8–10 weeks) were anaesthetised by intraperitoneal administration of a mixture of xylazine (10 mg.Kg⁻¹) and ketamine (50 mg.Kg⁻¹). Then, 30 µL of bacterial suspension (5 × 10⁶ for PAO1 and 1 × 10⁷ for IHMA/IHMAΔA) were deposited in the animal's nostrils.

To study survival rates, mice were inspected six times per day; moribund mice were considered dead and were euthanized by CO₂ inhalation. Experiments were stopped at day 5.

To investigate various infection parameters, mice were euthanized at 8 or 18 hpi, as indicated. Lungs, liver, and spleen were isolated and homogenised in PBS with a Polytron. When indicated, anakinra (Kineret from SOBI laboratories), was injected IP at 10 mg/kg 1 hr prior infection and then daily.

These in vivo experiments were performed only once; the results obtained with all mice used in these experiments are presented in the figures.

4.6 | Analysis of organ homogenates

The presence of caspase-1 was examined by Western blotting using an appropriate antibody (Santa Cruz Biotechnology; sc-56036). Bacterial

colony-forming units (CFU) were determined by plating serial dilutions and counting colonies on *Pseudomonas* Isolation (Difco) Agar plates. The CFU were calculated for the total organ. IL-1α, IL-1β, IL-6, IL-10, IL-17a and TNF-α levels were measured using the corresponding ELISA kits from BioLegend; KC levels were determined using the ELISA kit from R&D Systems and IL-18 by the LEGENDplex bead-based immunoassay from BioLegend. IL-1β was analysed by Western blotting as above, and membranes were reprobed with an anti-β-actin antibody to allow signal normalisation (Sigma-Aldrich A1978). MPO activity was determined using the Myeloperoxidase Fluorometric kit from Enzo Life Sciences. All dosages were performed once in triplicate.

4.7 | Analysis of BALs

BALs were collected at 18 hpi by flushing the lungs of euthanized and intubated mice with 0.5 mL PBS (three times). The fluids collected were pooled, centrifuged and the supernatants were aliquoted, snap-frozen and stored at –80°C. Protein concentration in the supernatants was measured using the BCA kit from Thermo Scientific. The presence of β-actin was determined by Western blot analysis, as above. Lactate dehydrogenase (LDH) activity in BAL supernatants was assayed using the Cytotoxicity Detection kit (Roche) and IL-1α was measured by ELISA as above. Dosages were performed once in triplicate.

4.8 | Transmission electron microscopy

For electron microscopy, mice euthanized at 18 hpi were rapidly perfused intracardially with NaCl 0.9%/heparin 20 U/mL and then with a fixative solution (paraformaldehyde 2%/glutaraldehyde 2%). Lungs were minced in small pieces (about 1 mm³) and prepared for electron microscopy as previously described (Bouillot et al., 2017). For transmission electron imaging, 80-nm sections, collected on formvar-coated copper grids, were observed under an FEI Tecnai G2 Spirit BioTwin transmission electron microscope operating at 120 kV using an Orius SC1000B CCD camera. Two mice were analysed per bacterial strain.

4.9 | Histological analysis

The histology of infected organs was examined. Organs were fixed, paraffin-embedded, and sections were prepared and stained with haematoxylin-eosin for morphological examination. Bacteria were labelled with an anti-LPS antibody (Acris Antibodies BP2235) and nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Slides were observed under an Axioplan Zeiss microscope.

4.10 | Mouse neutrophil isolation

Neutrophils were isolated from bone marrow using established procedures (Swamydas & Lionakis, 2013). Briefly, mice were euthanized

with 180 mg/Kg of Dolethal. Blood was collected by cardiac puncture and the serum was recovered. Bone marrow was harvested from femurs and tibias to isolate neutrophils. Neutrophils were purified on a Ficoll Paque Plus gradient (GE Healthcare). Purity (>90%) was determined by fluorescence microscopy after labelling nuclei with Syto 24 (Life Technology).

To assess NETosis, purified neutrophils were incubated for 3 hr with bacteria, then fixed and labelled with Hoechst and anti-H2AX antibodies (Millipore 07-727). The experiment was performed twice.

To monitor the phagocytic capacity of neutrophils, purified neutrophils were first labelled with the fluorescent DNA binding probe DRAQ5 (Life Technology) to exclude any non-lysed erythrocytes from the analysis. Then, cells were incubated with *E. coli* pHrodo Green (Life Technology) for 1 hr at 4 or 37°C in presence of 5% autologous serum. They were subsequently labelled at 4°C with an anti-Ly6G antibody conjugated with phycoerythrin (Becton Dickinson, 551461). Finally, cells (30,000) were analysed using a BD FACSCalibur, and the pHrodo Green fluorescence intensity was monitored on DRAQ5+/Ly6G+ cells (i.e. neutrophils).

4.11 | Time-lapse microscopy and data analysis

Kinetic experiments were performed on an IncuCyte live-cell microscope (Sartorius). Purified neutrophils were exposed to bacteria in a chamber at 37°C with CO₂, and phase-contrast images were automatically acquired every 15 min for up to 5 hr to monitor the progressive loss of intact neutrophils. Images were analysed with ImageJ software to determine the number of intact neutrophils at each time point. Nine images were collected per condition. The experiment was performed three times.

4.12 | Statistical analyses

Statistical analyses were performed using GraphPad Prism software (v. 7.00). All quantitative data were analysed by applying Shapiro-Wilk's normality test. In order to not overload the figures and to show only the relevant comparisons, we compared (a) the WT versus KO data for each strain and (b) the data between strains only in WT mice. The comparison of data between strains for the KO mice were not calculated. Significance of differences between data for WT and KO mice was determined using Student's or Mann-Whitney's tests (as indicated in the legends), depending on the result of the normality test. Significance of differences between data from the three strains and non-infected control in WT mice was determined using a one-way analysis of variance (ANOVA) test, as all data from WT mice had a normal distribution; $p < .001$ for all data. For multiple comparisons, a Tukey's test was used. (*) indicates a $p < .05$ and n.s. indicates a non-significant difference. For clarity, significance relative to uninfected controls is only shown when non-significant.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Stéphanie Bouillot participated to most experiments of the study. Stéphane Pont performed the experiments on neutrophils and made some dosages. Benoit Gallet and Christine Moriscot did the electron microscopy work. Vincent Deruelle performed some in vivo analyses. Ina Attrée contributed to the conceptualisation of the project. Philippe Huber participated to most experiments and wrote the manuscript.

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Chapter VIII

An unusual community-acquired invasive and multi-systemic infection due to ExoU- harboring *Pseudomonas aeruginosa* strain: Clinical disease and microbiological characteristics

1) Research Article

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P. aeruginosa is usually considered as an opportunistic pathogen, meaning that it mainly affects compromised individuals like cystic fibrosis or immuno-depressed patients, and is unable to infect immunocompetent individuals. However, in rare cases, community-acquired infections can be observed in patients with no underlying comorbidities. This manuscript describes such a clinical case caused by a hyper-virulent strain secreting the very potent ExoU toxin.

This strain, named YIK, was isolated from clinicians of the Tenon Hospital (Paris) and sent to us to better characterize its virulence characteristics. We could identify that this hyper-virulent isolate, which almost caused the patient to die, uses its type 3 secretion system to secrete the phospholipase ExoU, at the origin of its important cytotoxicity.

In this project, I performed all the experiments aiming at characterizing the cytotoxicity of the strain.



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Short Communication

An unusual community-acquired invasive and multi systemic infection due to ExoU-harboring *Pseudomonas aeruginosa* strain: Clinical disease and microbiological characteristics



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Abstract Community-acquired *Pseudomonas aeruginosa* infections are rare. Most cases involve patients either with underlying immunosuppression or structural chronic lung diseases. We report here an atypical case of a severe community-acquired invasive infection due to a hypervirulent ExoU-producing strain, in an immunocompetent patient.

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Introduction

Community-acquired *Pseudomonas aeruginosa* (*P. aeruginosa*) infections are rare. Most cases involve patients either with underlying immunosuppression or structural chronic lung diseases, such as bronchiectasis. The virulence factors play an important role that may lead to acute lung injury, bacteremia, sepsis and invasion of tissues. *P. aeruginosa* possesses a virulence mechanism known as the type III secretion system. The type III secretory toxin, ExoU is a major virulence factor associated with poor clinical outcome. We report here an atypical case of a community-acquired invasive infection due to a hypervirulent ExoU-producing strain, in an immunocompetent patient.

Case report

A 49-year-old man was admitted to the intensive care unit (ICU) of our hospital for septic shock. He had no previous underlying disease, except being a past smoker; he was not taking any medication and had no relation with any type of healthcare resources. Three days before ICU admission, he returned to France by car from Turkey where he had stayed by the sea for 1 month. During the trip, he reported diffuse abdominal and back pain with nausea, vomiting and diarrhoea associated with chills. He was referred to the Emergency department of our hospital. Glasgow Coma Scale was 15/15, blood pressure 75/50 mmHg, heart rate 100 beats/min, respiratory rate 22/min, room air pulse oximetry 98% and core temperature 37.1 °C. Physical examination revealed diffuse abdominal pain, mainly in the right upper quadrant of the abdomen, and subcutaneous erythematous nodules on the torso, pelvis and legs (Fig. 1A). Laboratory tests on ICU admission were as follows: leukocytosis 14×10^9 cells/L (N $4\text{--}10 \times 10^9$ /L), polymorphonuclear neutrophils (PMNs) 11.8×10^9 (N $1.5\text{--}7 \times 10^9$ /L), haemoglobin 11.6 g/dL (N 12–18 g/dL), platelet count 113×10^9 cells/L (N $150\text{--}400 \times 10^9$ /L), serum creatinine 307 $\mu\text{mol/l}$ (N 60–120 $\mu\text{mol/l}$), serum urea 19.5 mmol/L (N 3–7), lactate 3.2 mmol/l (<2 mmol/l), prothrombin time 60% (N 70–120%), serum bilirubin 19 $\mu\text{mol/l}$ (N < 17), serum aspartate aminotransferase 544 IU/L (N < 35), serum alanine aminotransferase 299 IU/L (N < 43), and CRP 476.3 mg/L (N < 5). Chest X-ray and computed tomography (CT) scan showed a left-upper

alveolar infiltrate with excavation, suggestive of a necrotizing pneumonia (Fig. 1B), while abdominal and pelvic CT scans were unremarkable.

Fluid expansion and catecholamines were administered promptly. Empirical antimicrobial therapy including cefotaxime, spiramycin and linezolid was initiated. The patient rapidly developed acute respiratory failure requiring high-flow nasal cannula oxygen therapy.

A bronchoalveolar lavage (BAL) was performed on day 1, revealing neutrophilic alveolitis (200×10^3 /ml, 59% PMNs) without any microorganism on Gram stain. Skin biopsies were also performed on day 1 on subcutaneous erythematous nodules. Pathological examination revealed lesions of neutrophilic panniculitis associated with lesions of vasculitis with thrombosis suggestive of ecthyma gangrenosum. Finally, a wild-type *P. aeruginosa* yielded on BAL, blood, and urine cultures on day 2, and all the strains had the same profile of antimicrobial resistance. Viral and fungal cultures of BAL were negative. Empirical antibiotics were stopped, and ceftazidime and ciprofloxacin were initiated on day 3.

A binocular blurring followed by a scotoma of the right eye occurred on day 1. A brain MRI was performed and found no abnormalities. The fundus examination revealed subretinal nodules (Fig. 1C) suggestive of endogenous endophthalmitis. Two intravitreal injections of ceftazidime were administered on day 8, resulting in a retinal atrophy scarring on day 15. A functional disability of the left leg associated with pain occurred on day 4, suggestive of cruralgia. There were no signs of spondylodiscitis or discoradicular conflict on spinal MRI. A repeat abdominal and pelvic CT scan revealed a nodular hypodensity of 12 mm at the femoral insertion of the left ilio-psoas muscle. Finally, a (18F)-fluorodeoxyglucose positron emission tomography was performed and identified several areas of increased metabolic activity within the lungs, the trochanteric insertion of the left psoas, the prostate and the subcutaneous nodules. A trans-oesophageal echocardiography ruled out an infectious endocarditis.

The patient gradually improved with a rapid decrease of oxygen therapy and a complete recovery of renal and liver functions. It should be noted that cutaneous lesions co-existed at different stages of development, some of which had evolved towards gangrenous ulcers like ecthyma gangrenosum. However, the right eye scotoma still persisted. Finally, the patient was discharged from hospital on day 24.

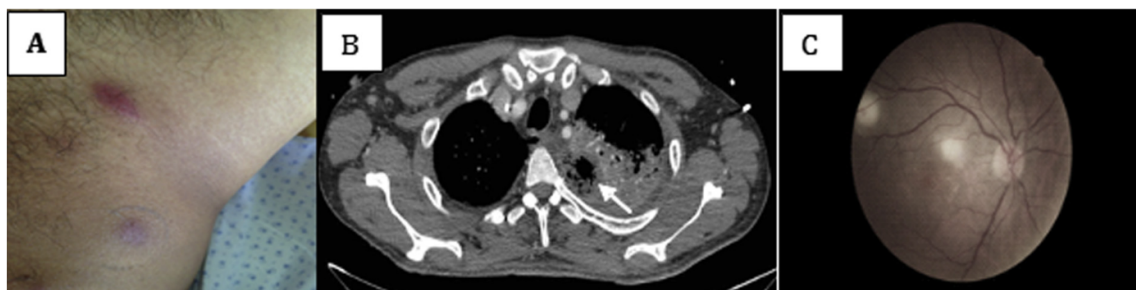


Figure 1. Multiple necrotizing organ damages due to *P. aeruginosa*. **1A.** Erythematous nodules of the left side of the pelvis. **1B.** Section of lung CT scan showing air-space consolidation with necrosis (arrows) and air-bronchogram of the left upper lobe. **1C.** Colour fundus photograph of the left eye with two areas of retinitis.

Additional investigations were performed to search for an immunosuppressive status, including HIV testing, neutrophils count and functional activity, serum protein electrophoresis, and biological dysimmunity. The results of these tests were all negative, and there was no underlying diabetes.

Microbiological findings

The strain of *P. aeruginosa* (YIK) was investigated further. This isolate turned out to belong to a quite rare sequence type, ST137 (<https://pubmlst.org/paeruginosa/>), and like the hypervirulent strain PA14,¹ to code for an exotoxin named ExoU. Considering the impressive ability of this strain to invade multiple organs in an immunocompetent individual, we examined the capacity of the strain to induce epithelial cell death *in vitro*. The A549 cells were incubated with YIK and laboratory strains PAO1 (that

produces the less potent exotoxin ExoS⁺) and PA14 (ExoU⁺), in presence of propidium iodide (PI) to monitor the kinetics of membrane permeability over 300 min post-infection. The cytotoxicity is presented as an increase in fluorescence intensity (AU) and the calculated area under curve (AUC) as described in the material and methods section. In accordance with the fact that ExoS⁺ strains do not trigger necrosis,² PAO1 did not induce any significant incorporation of PI within epithelial cells within time lapse of 300 min, whereas YIK was the most cytotoxic strain (Fig. 2A and B), causing even more cell death than the ExoU-positive strain PA14. To assess whether the cytotoxicity induced by YIK was dependent on an active type III secretion system (T3SS), we used an immune serum directed against T3SS protective antigen PcrV, which is an essential component of this secretion machinery. This serum was previously shown to inhibit *P. aeruginosa* induced haemolysis.³ Indeed, as shown in Fig. 2C and D, the cytotoxicity of the YIK strain towards A549 cells was significantly inhibited in the

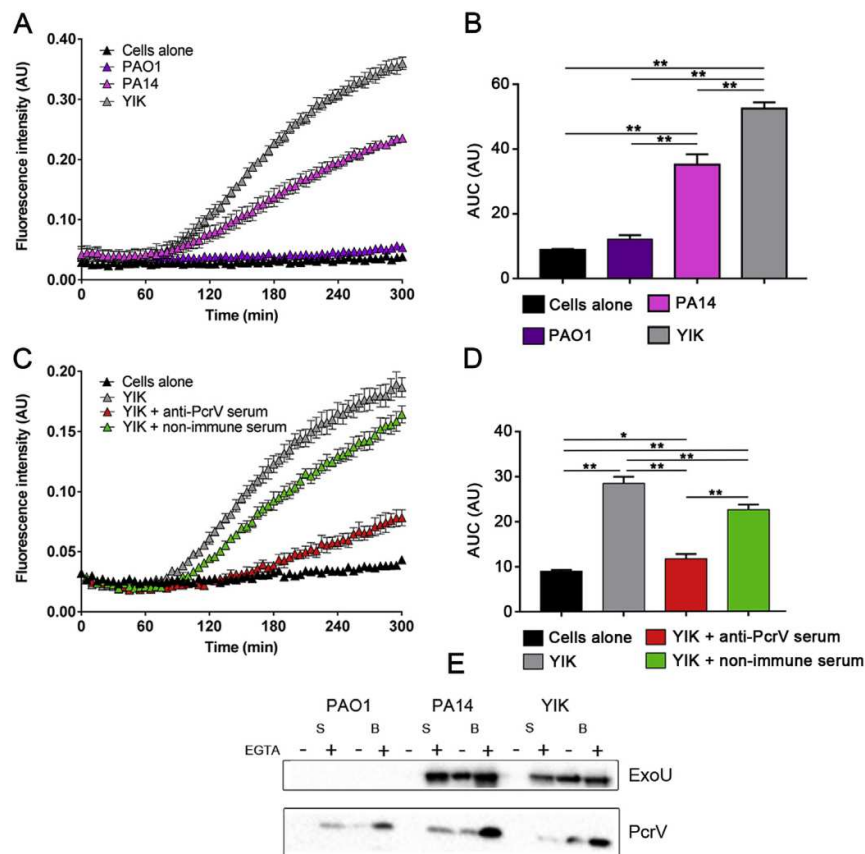


Figure 2. *P. aeruginosa* YIK is cytotoxic on epithelial cells by using the T3SS effector ExoU. **2A.** Kinetic of propidium iodide (PI) incorporation in A549 cells in presence of PAO1 (ExoS⁺), PA14 (ExoU⁺) or YIK, at a MOI of 10. At each time point, data represent mean \pm SD of triplicates. Results are representatives of three independent experiments. **2B.** Cytotoxicity curves in A. represented as area under curves (AUC). ANOVA, $p < 0.001$; Holm-Sidak's post-hoc test: $**p < 0.001$. **2C.** Kinetic of PI incorporation in A549 cells infected with YIK, in presence of either immune serum directed against the protective antigen PcrV (anti-PcrV) or non-immune serum. At each time point, data represent mean \pm SD of quadruplicates. Results are representatives of three independent experiments. **2D.** Cytotoxicity curves in C. represented as area under curves (AUC). ANOVA, $p < 0.001$; Holm-Sidak's post-hoc test: $*p = 0.007$, $**p < 0.001$. **2E.** Detection of PcrV and ExoU. PAO1, PA14 and YIK culture supernatants (S) or bacterial cells (B) were analysed by immunoblotting using specific antibodies raised against PcrV and ExoU. T3SS was induced where indicated by adding EGTA and MgCl₂, as described in Materials and Methods.

presence of anti-PcrV serum. The kinetics and the inhibition of cytotoxicity on A549 cell by the anti-PcrV serum confirmed that the YIK induced cell death was caused by the well-described T3SS effector ExoU.⁴ To confirm the synthesis and secretion of ExoU by YIK, we performed immunodetection of secreted proteins by different strains under conditions where T3SS is induced by chelation of calcium (+EGTA). ExoU was readily detected in whole bacteria (B) and supernatants (S) of PA14 (positive control strain) and YIK, and was absent from PAO1, that lacks gene *exoU*. PcrV was detected in all three strains. In conclusion, these experiments show that the YIK strain is highly cytotoxic on epithelial cells in an ExoU-dependent manner.

Materials and methods (online supplement)

Discussion

This observation is remarkable for several reasons. First, the illness was community-acquired and occurred in a subject with no known immunodeficiency. Second, the illness was severe with septic shock and direct multiple necrotizing organ damages (necrotizing pneumonia, muscular abscesses, ecthyma gangrenosum, retinal involvement and prostatic localisation). Third, an unusual virulent strain of *P. aeruginosa* with ExoU positive phenotype was identified.

In a retrospective series of 39 cases of community-acquired *P. aeruginosa* bacteraemia, Kang et al.⁵ reported that the primary site of infection was the lung (23% of cases), followed by the biliary (20%) and urinary (12%) tracts. The initial presentation was associated with septic shock in 38% of cases, overall mortality rate was 38.5% and only 2 patients were immunocompetent. The overall incidence of community-acquired *P. aeruginosa* bacteraemia is estimated at 1 to 1.3/100,000,⁶ with a greater incidence in men. The associated ocular damage due to *P. aeruginosa* is also very atypical and few cases have been reported in immunocompetent patients.⁷ In children, several severe community-acquired cases of *P. aeruginosa* infection have been reported in the context of Shanghai fever. Fever and diarrhoea are the most common clinical presentation and ecthyma gangrenosum occurs in more than 50% of cases.⁸ In our case, there was no sign of enteritis, despite the fact that the patient suffered initial digestive symptoms.

Virulence of *P. aeruginosa* is multifactorial.^{9,10} Among the factors that *P. aeruginosa* may produce during infection, ExoU is of major clinical importance because of its high cytotoxic and procoagulant activities, as well as its role in vascular hyperpermeability, platelet activation and thrombus formation.¹¹ ExoU is a phospholipase directly injected into the eukaryotic cells by the needle-like T3SS of *P. aeruginosa*, with subsequent cell death and tissue necrosis. Infections related to ExoU-producing strains are associated with more severe clinical symptoms and poorer outcomes, as compared with ExoS-positive (ExoU-negative) isolates.^{11,12} They are also responsible for acute lung epithelial injury, sepsis, and corneal lesions.¹³ In 328 *P. aeruginosa* clinical and environmental isolates collected worldwide, Pirnay J-P et al. reported that 73% of isolates harboured the *exoS* gene, which was indicative of an

invasive phenotype, and that 23% of isolates harboured the *exoU* gene, which was associated with virulence and disease severity.¹⁴ There was no difference in the distribution of genotypes between clinical and environmental isolates, except for the cystic fibrosis (CF) isolates that exhibited almost exclusively the *exoS*+/*exoU*- genotype, which might be required for the process of a bronchial colonization in CF lungs. The examination of the Shanghai fever strains showed that 59% of them were carrying an ExoU strain, and 41% an ExoS strain. Shanghai fever isolates with *exoU* were significantly more cytotoxic than isolates with *exoS*.⁸

Innovative therapeutic strategies, based on T3SS-targeting antibodies or inhibitors, have been proposed over the last decade to combat nosocomial strains of *P. aeruginosa*.^{11,15} As illustrated here, such novel approaches could also be valuable in the treatment of life-threatening community-acquired infections.

Conclusion

We report a rare case of a particularly severe lower respiratory tract infection with septic shock and multiple direct necrotizing organ damages due to a highly cytotoxic strain of *P. aeruginosa* exhibiting an ExoU-phenotype. Physicians should be aware of the possible occurrence of such an infection in immunocompetent hosts, to early target anti-pseudomonal antimicrobial treatments.

Ethical approval and consent to participate

Not applicable.

Consent to publish

The patient gave a written consent for publication of his potentially-identifying information (including individual details and images).

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MD and MF drafted the manuscript. AE and GV were responsible for the clinical management and therapy. PP, CV, FC and SP performed the investigation of the strain of *P. aeruginosa*. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmii.2019.06.008>.

General discussion and perspectives

Behavior of *Pseudomonas aeruginosa* in human whole blood

The interaction between *P. aeruginosa* and the innate immune system of the blood is still poorly understood, while BSI related to this pathogen are associated with particularly poor outcomes. *P. aeruginosa* has been shown to evade killing by purified neutrophils and to withstand complement-mediated lysis in serum (Dacheux et al., 2000, 1999; Dzvova et al., 2017; Hallström et al., 2015; Priebe et al., 2004; Usher et al., 2002), however within the circulation it is still unclear which of these strategies are used by the bacteria to persist and disseminate.

We observed that rather than being cytotoxic toward circulating phagocytes, the most resistant strains in the blood were resistant to the lytic action of the complement. Even though we could not observe differences between survival of T3SS⁺ (either ExoS⁺ or ExoU⁺) and ExlA⁺ strains, we tested three isolates of each type. However, the number of tested strains should be increased to have a more significant evidence that the nature of the major toxins affects or not bacterial survival in this context. Likewise, to confirm that cytotoxicity is not essential for the survival in whole blood, behavior of non-cytotoxic *exsA* (the main activator of the T3SS genes) and *exlA* deletion mutants should be compared to WT. Our work and the one of others (Thanabalasuriar et al., 2017) show that in the circulation, PMNs are not efficient at killing *P. aeruginosa*, while they are essential to fight this pathogen within tissues. Neutrophil recruitment out of the vasculature prime these cells through various stimuli (e.g C5a, IL-8, diapedesis), enhancing their phagocytosis capacity and ROS production, likely at the origin of the discrepancy that we pointed out (Miralda et al., 2017). It would be interesting to assess if PMN priming in the resting blood prior to the addition of *P. aeruginosa* could increase their bactericidal capacities. We also observed that the majority of the strains were efficiently detected by blood leukocytes as shown by the important cytokine release. However, it is known that the strain PA7 through the activity of PumA (a TIR domain containing protein) is able to prevent NF- κ B translocation to the nucleus of epithelial cells (Imbert et al., 2017), we were thus expecting an inhibition/decrease of the cytokine release by this isolate. The fact that PumA is not efficient in the blood should be confirmed by assessing the cytokine release induced by the WT and Δ *pumA* strains. Additionally, these experiments could be performed on different cell types (e.g neutrophils, monocytes) to assess if this effector is also efficient on immune cells.

We were also able to demonstrate that to prevent complete elimination in human plasma, complement-sensitive strains of *P. aeruginosa* and other human pathogens formed a subpopulation of “complement evaders”, phenotypic variants withstanding the lytic action of this system but unable to transmit their tolerance to their progeny upon stress removal. Nonetheless, the mechanism by which these cells originate has not been elucidated yet, and many unanswered questions concerning this population still persist. Complement evaders were characterized in plasma, but were also observed in whole blood, meaning that they were able to resist internalization by circulating leukocytes. As we observed that blood phagocytes are only efficient through opsonophagocytosis, we hypothesize that evaders are able to prevent C3b binding at their surface. This would prevent at the same time complement activation at their surface with subsequent lysis and opsonophagocytosis. Thus, the level of C3b and MAC binding to evaders versus initial population will be assessed by FACS. However, this will be possible only if surviving evaders can be differentiated from dead bacteria. During this project, we tried to label killed bacteria using DNA marker propidium iodide (PI), without obtaining any signal suggesting that either PI is expelled or bacteria are emptied of DNA. If DNA is lost upon MAC-mediated pore formation, evaders might be identified using a cell-permeant DNA-binding dye (e.g SYTO 24 green) following the incubation in plasma. Also, GFP⁺ cells likely lose GFP signal upon complement-mediated killing, another way to spot evaders which would still harbor a green fluorescence following the challenge. FACS could be also used to sort evaders in order to perform RNA-Seq and identify if some known complement-resistance factors are over-expressed, typically proteases or exopolysaccharides, known to interfere with C3b binding at the bacterial surface. This was done with intramacrophagic *S. aureus* persisters (Peyrusson et al., 2020), and allowed to determine that these bacteria undergo a global transcriptomic reprogramming, following induction of the SOS, heat shock, cell wall stress and stringent responses.

From our data, we concluded that complement evaders are generated through an energy-dependent process, and that they do not present an atypical lag-time before starting to grow following the stress. However, we were not able to assess their growth status during incubation in plasma. To that aim, we could perform a fluorescent dilution experiment, using either a fluorescent stable dye or an inducible GFP construct. Monitoring fluorescence before and after the incubation in plasma will allow to determine the division rate of evaders. Another unknown

feature of evaders is whether they appear stochastically prior the stress, or if the phenotype is triggered by the contact with plasma. This question could be addressed by creating reporter constructions to monitor the expression of known complement-protective factors or stress response regulators, to assess if already before the challenge, a rare subpopulation can be identified thanks to an abnormal overexpression of one of these genes. This experiment could be performed in a second step, following the RNA-Seq, which could allow selecting few potentially interesting genes. Finally, we do not know if complement evaders are also resistant to antibiotics. It was shown that persists to certain antibiotics were also resistant to complement-mediated lysis in uropathogenic *E. coli* (Putrinš et al., 2015). Because plasma protein binding of certain antibiotics is known to modify importantly the level of active (unbound) antimicrobial molecule, minimal inhibitory concentrations should be determined in heat-inactivated plasma. Then, bacteria should be challenged in normal plasma with or without antibiotic to assess the presence of evaders in these conditions.

New bacterial factors involved in the interaction between *Pseudomonas aeruginosa* and the complement system

To identify bacterial factors involved in complement evasion and/or resistance of *P. aeruginosa*, we performed Tn-Seq experiment using plasma as a challenge. As proof of concept, the first screen was performed with the Tn library constructed in the strain IHMA87 that is plasma sensible and form evaders. We identified several known and unknown factors whose dysregulation was beneficial for the strain survival in plasma.

Inactivation of genes involved in the biotin and purine biosynthesis significantly heightened bacterial survival upon contact with complement (6 and 50 fold-increased survival, respectively). Due to its cofactor activity for carboxylases essential in fatty-acid synthesis, biotin deficiency is likely to affect the lipid composition of the bacterial membrane, thus influencing MAC insertion. In the Gram positive bacterium *Lactococcus lactis*, spontaneous auxotrophs for guanine were shown to be resistant to lysozyme-mediated lysis, as they harbored a thicker and more rigid cell wall (Solopova et al., 2016), while in *S. aureus*, the same incapacity to synthesize guanosine induces an increased secretion of proteases known to degrade complement proteins C3 and C5 (Jusko et al., 2014; King et al., 2018). Both mechanisms could

positively affect the survival for *P. aeruginosa* in plasma, and should thus be investigated by assessing the ability of our mutants of interest to withstand other types of membrane-targeting stresses (e.g. detergents, antimicrobial peptides) and their proteolytic potential against complement proteins. Electronic microscopy could also be used to compare the membrane structure of the mutants compared to the parental strain. Finally, RNA-Seq on the $\Delta purD$ and $\Delta bioB$ background could provide information on the dysregulated genes in these mutants, some of them possibly directly involved in the interaction with the complement.

It appears that *de novo* synthesis of biotin and purines are needed for *P. aeruginosa* to adapt to the nutritious condition of serum (Carfrae et al., 2020; Poulsen et al., 2019; Samant et al., 2008). In addition, cells unable to synthesize purines have predicted decreased fitness in the lung environment (Poulsen et al., 2019). Both purine and biotin auxotrophs were associated with attenuated virulence in mice models of infection; the relevance of our findings should thus be evaluated in a physiological infectious context. Absolute requirement of biotin and purine to multiply in the host implies that auxotrophs for these metabolites will colonize niches in which these compounds are available, like in the gut (Qi et al., 2017; Said, 2009). Extracellular DNA (either bacterial or from eukaryotic cells) in the lung of CF patients has also been recognized as a potent source of purines for auxotroph *P. aeruginosa* (Kumar et al., 2019). These bacteria will thus only take advantage of their auxotrophy (regarding complement protection) upon dissemination, through the vasculature, between two niches supporting their growth. Because purine and biotin biosynthesis appear to be required for the pathogen to multiply in infection-relevant conditions, and because humans are auxotroph for biotin, these two pathways have been proposed to be valuable targets for antibiotics. This might indeed be an interesting path, but should require the verification that these treatments do not enhance the ability of bacteria to resist complement-mediated lysis.

During the screen, we found that overexpression of three genes encoding proteins of unknown function increased the ability of the bacteria to survive in plasma compared to the wild type IHMA87 strain (1000 fold-increased survival). This brought us to name these genes ‘‘serum resistant genes’’ or *srgABC*. Regulation of the expression of this operon seems to be dependent on quorum sensing (Nalca et al., 2006; Skindersoe et al., 2008), but the function of these three small proteins (size < 10kDa) have never been studied. The first protein SrgA encoded by the operon is predicted to carry an N-terminal cleavable Sec-signal peptide,

meaning that it might be translocated to the periplasm through the Sec machinery. SrgB and SrgC are predicted to be localized at the inner membrane. Interestingly, IHMA87_Tn-*srg* displayed mucoid phenotype on plates and the levels of alginates were increased compared to the parental strain. The studies to elucidate link between alginate biosynthesis, complement resistance and the *srgABC* operon is underway in the laboratory. However, we can hypothesize that the *srgABC* gene products are positive regulators of alginate synthesis. It may be interesting to investigate whether the *srgABC* operon is over-expressed in CF isolates displaying highly mucoid phenotype. Modification of LPS is most of the time observed in mucoid isolates, with a loss of OSA expression, rendering them complement-sensitive. We doubt that OSA expression is negatively affected in this mutant, as it was serotypeable and has undistinguishable O11/12 serotype as the parental strain. However, its LPS structure should be examined, notably to assess if this mutant harbors increased amount of long O-antigen chain length protective toward complement (Kintz et al., 2008), as positive regulation of alginate biosynthesis has been shown to affect OSA chain length through a transcriptional cross regulation (Cross and Goldberg, 2019). Also, the mechanisms by which this mutant evade complement-mediated lysis should be addressed by assessing the binding of C3b and MAC at its surface, along with its ability to activate each of the three complement pathways, compared to parental strain. The ability of the mutant to withstand other membrane-targeting stresses should also be investigated.

Conclusion

In this work we describe for the first time the existence of complement evaders, likely enabling various complement-sensitive Gram-negative pathogens to persist in the blood. To tackle these cells, a comprehensive analysis should be undertaken to identify underlying mechanisms responsible for their formation. We also demonstrate that different *P. aeruginosa* strains display variable levels of complement susceptibility. Knowing that the interaction between the complement system and a bacterial species is affected by strain-specific traits, a strain-wide Tn-Seq screen in human plasma may be necessary to provide the global complement resistome of *P. aeruginosa*. Finally, the genome-wide screen performed in this study allowed to identify new bacterial factors involved in the interplay of *P. aeruginosa* and

the complement system, notably a previously uncharacterized three-genes operon which products promote bacterial survival in human plasma and appearance of a mucoid phenotype. The exact mechanism mediating enhanced survival will be deciphered, along with the role of these genes in the overproduction of alginate.

Material and Methods

CFU measurement in HIP or normal plasma

Bacterial viability was assessed in plasma exactly as described in the Methods of the research article presented in Chapter I. To inactivate complement, plasma was incubated 30 min at 56°C. Following the treatment, tubes of HIP were centrifuged 5 min at 5000 g and the plasma was filtered on a 0.2 µm filter, to eliminate the precipitates formed during heating.

Bacterial visualization in HIP

P. aeruginosa expressing GFP was incubated in HIP as for the CFU measurement. Every 15 min, 10 µL of the suspension were spotted on a 1% agarose pad prepared in PBS. After absorption of the plasma, the pad was placed in a 1-chamber LabTek slide (Nunc) and observed using a 50x or 100x oil immersion objective under the microscope (Axioplan, Zeiss). Pictures were taken using the camera RT KE Spot (Spot Imaging).

Sonication allowing the individualization of aggregated bacteria

Following 1h incubation in plasma, 100 µL samples were transferred in 200 µL PCR tubes and placed in ultrasonic bath of the sonicator Q700 (Qsonica). Following the manufacturer instructions, tubes were placed at 2 mm from the probe, to increase the efficiency and reproducibility of the treatment. Sonication was performed at 6°C for 45 sec at 10% intensity.

Statistical analysis and graph plotting

They were done exactly as described in the Methods of the research article presented in Chapter I. Mann-Whitney U test was used to compare two data sets not following log-normal distributions.

Bacterial strains, plasmids and primers

Strain	Genotype	Reference
IHMA-GFP	IHMA87 with constitutive expression of GFP, carrying miniCTX-pX2- <i>gfp</i> (Tc ^R)	This study
PA14-P _{<i>algU</i>} - <i>gfp</i>	PA14 carrying, at the <i>attB</i> site on its chromosome, the promoter region of the <i>algU</i> operon cloned upstream of <i>gfp</i>	
PA14-P _{<i>rpoS</i>} - <i>gfp</i>	PA14 carrying, at the <i>attB</i> site on its chromosome, the promoter region of the <i>rpoS</i> gene, upstream of <i>gfp</i>	
PA14Δ <i>bioB</i>	PA14 strain carrying the deletion in <i>bioB</i>	
PA14Δ <i>purD</i>	PA14 carrying the deletion in <i>purD</i>	
IHMAΔIHMA87_01573	IHMA caring the deletion in IHMA87_01573 (<i>srgA</i>)	
IHMAΔIHMA87_01574	HMA carrying the deletion in IHMA87_01574 (encoding the acetyltransferase)	

Table 5: List of the strains used in this study (in addition to bacterial strains described in published or submitted manuscripts)

Plasmid	Property	Reference
pUC57	Vector containing the proper flanking regions to perform clean deletions	Genewiz
pEXG2	Suicide plasmid without replicative origin in <i>P. aeruginosa</i> , used for allelic exchange (<i>sacB</i> , Gm ^R).	(Rietsch et al., 2005)
miniCTX1	Integrative plasmid at the <i>attB</i> site on the chromosome (Tc ^R)	(Hoang et al., 2000)
miniCTX- <i>gfp</i>	miniCTX1 carrying <i>gfp</i> (Tc ^R)	Lab collection
miniCTX-PX2- <i>gfp</i>	miniCTX1 carrying <i>gfp</i> under the control of the constitutive PX2 promoter (Tc ^R)	
pRK2013	Helper plasmid with conjugative properties (Kn ^R)	(Figurski and Helinski, 1979)
pRK600	Helper plasmid with conjugative properties (Cm ^R)	(Kessler et al., 1992)
pCR-Blunt II-TOPO	Commercial cloning vector (Kn ^R)	Invitrogen
pFLP2	Plasmid allowing the excision of the integrated miniCTX1 backbone thanks to the FLP recombinase (<i>sacB</i> , Cb ^R)	(Hoang et al., 1998)

Table 6: List of plasmids used in this work.

Sequence (5' => 3')	Use
mut-ACTase-F0-F: CGGAGGATGAAGACGCGCG mut-ACTase-R0-R: ATGTCCAGGCCTGTTCGTCG	Deletion in <i>IHMA87 01574</i>
mut-srg1-F0-F: GGTGACCCTGGTGGCCGA mut-srg1-R0-R: CAGGCTGGGATCGAAGCGA	Deletion in <i>IHMA87 01573</i>
purD_F0-F: GAGCAGGAGATCCACGACCT purD_R0-R: ACGTTGCCTTCCTCGGCCAT	Deletion in <i>purD</i>
bioB_F0-F: GGAAAGTGGTTGGTCGGAAG bioB_F0-R: TTGAGGCGGTCCCTCCAGCA	Deletion in <i>bioB</i>
opmQ_F0-F: GACTACGAGCTGACCAACAAC opmQ_R0-R: CAGGGTAATCAGCGCCTCAT	Deletion in <i>opmQ</i>
algU-EcoRI-F: CCGAATTCCGCCCTTGCTGAGGACG algU-R-BamHI-R: CCGGATCCGGAGGAGCTTCGAGCGTCC	Amplification in P_{algU}
rpoS-EcoRI-F: CCGAATTCCCTGCCGCTGCGCGGC rpoS-BamHI-R: CCGGATCCACCGGCGTATACAACGGTCC	Amplification in P_{rpoS}
Forked adapter (short): TACCACGACCA-NH2 Forked adapter: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGTCGTGGTAT	DNA library preparation for NGS
Illumina primer P5: AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGA CGCTCTTCCGATC	
Illumina primer P7: CAAGATCAAGCAGAAGACGGCATAACGAGAT- index-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC	
Transposon specific: CACAGGAAACAGGACTCTAGAGG	
Rnd1a: GGCCACGCGTCGACTAGTAGNNNNNNNNNCAGCAG Round1-pBT20_wen: GAAGCTGTGGTATGGCTGTGCAGG	Semi-random PCR (first step)
Round2-PA: GGCCACGCGTCGACTAGTAC Round2-pBT20_wen: CGCACTCCCGTTCTGGATAATGTT	Semi-random PCR (second step)

Table 7: List of primers and adapters used in this study

Generation of deletion mutants

DNA sequences flanking the region to be deleted were ordered from Genewiz. They were subcloned from the parental vector pUC57 to the recipient plasmid pEXG2. The product of this subcloning was used to transform TOP10 *E. coli* competent cells (Thermofisher). The product of the transformation was plated on LB agar containing Gentamicin and allowed to grow over night to select cells having received the pEXG2 plasmid carrying the fragments. Gm^R colonies were then screened by PCR to identify those carrying fragment of interest, which

were subsequently confirmed by restriction analysis. The deletion mutants were obtained by double recombination. To that aim, plasmids were transferred to *P. aeruginosa* by triparental mating, using either pRK2013 (for PA14) or pRK600 (for IHMA) as a helper plasmid, as previously described (Trouillon et al., 2020). Isolated colonies on selective medium were plated on LB without NaCl containing 10% sucrose, allowing to select bacteria in which the double homologous recombination happened. Counter-selected colonies were then screened by PCR to verify their mutant genotype, and the loss of resistance to Gentamycin was verified.

Creation of strains carrying reporter constructions

Using genomic DNA as a template, the 500 bp region located upstream to the 5'-UTR of the gene of interest was amplified by PCR using primers described in Table 7. The PCR fragments were inserted in pCR-Blunt II-TOPO (Invitrogen) and transformed in *E. coli* TOP10. Colonies carrying the plasmid with the fragment of interest were screened by PCR, and sequenced. Verified fragments were then subcloned in the miniCTX-*gfp* vector, upstream of the *gfp*. The *gfp* fusions were introduced in *P. aeruginosa* by triparental mating as described previously. The miniCTX backbone was excise thanks to the use of the pFLP2 plasmid, delivered to *P. aeruginosa* by transformation. Briefly, cells were rendered competent by incubation in cold CaCl₂ 75 mM, MgCl₂ 6 mM and glycerol 15% (w/v). Competent cells were then transformed with pFLP2 by heat shock (2 min at 39°C) (Hoang et al., 1998).

Microfluidic experiment

Microfluidic device used in the Van Melderens' lab was CellASIC ONIX Microfluidic System with the B04A-03 Microfluidic Plate (Millipore). Exponentially growing bacteria were resuspended in PBS at an OD₆₀₀=0.01. This suspension was loaded in the dedicated well. The culture chambers with trapped bacteria were perfused with LB medium allowing them to multiply for two hours, then human plasma was injected for six hours in order to select complement evaders, and finally LB was re-injected to allow regrowth of these surviving cells. Time-lapse microscopy was performed at 37 °C using a 100x oil immersion objective on an

inverted microscope (Axio Observer Z1, Zeiss). Phase-contrast images were recorded every 15 min with a Hamamatsu ORCA-Flash 4.0 digital camera and a Zeiss 38HE filterset.

Validation of the transcriptional fusions using flow cytometry

The general stress and stringent responses are both induced upon entry into stationary phase (Boes et al., 2008; Whiteley et al., 2000). Induction of P_{rpoS} -*gfp* and P_{uspL} -*gfp* was assessed by analyzing cells in stationary phase (overnight cultures) or exponentially growing bacteria ($OD_{600} = 1$, following 3h of culture in LB at 37°C). The SOS and envelope stress responses are activated following DNA damages and perturbations in the membrane, respectively. Induction P_{sulA} -*gfp* and P_{algU} -*gfp* was assessed following challenge with ofloxacin (SOS response) and the cell wall synthesis inhibitor fosfomycin (envelope stress) (Breidenstein et al., 2012; Wood et al., 2006). Overnight cultures were diluted in LB at an $OD_{600} = 0.1$ and allowed to grow in LB (37°C, 300 rpm) until OD_{600} reached 0.5. Bacteria were subsequently challenged with ofloxacin (10 µg/mL) or fosfomycin (500 µg/mL) for 45 min. For all four constructions, cells in LB were recovered by centrifugation (5 min at 5000 g), resuspended in PBS and analyzed for their GFP fluorescence intensity using a FACSCalibur (BD biosciences). 10000 events were analyzed.

Flow cytometry to assess the induction of bacterial stress responses in plasma

Following the incubation in plasma (heat inactivated or not), bacteria were recovered by centrifugation (5 min at 5000 g), plasma was eliminated and cells resuspended in PBS were analyzed for their GFP fluorescence intensity (emission filter 530/30) using an Attune NXT flow cytometer (Thermofisher). In each experiment, 10000 events were analyzed.

Screen in plasma of the IHMA87-Tn library

Culture condition and challenge

The mutant library was thawed on ice, and washed in LB to eliminate glycerol used as a cryoprotectant. Bacteria were allowed to grow in LB (37°C, 300 rpm) until the culture reached an OD₆₀₀ of 1. Exponentially growing bacteria were recovered by centrifugation (5 min at 5,000 g), resuspended in PBS supplemented with calcium and magnesium, and incubated in LB or pooled human plasma (90% final plasma concentration) at a concentration of 2.25x10⁷ CFU/mL for 3h at 37°C on a rotating wheel. Following this incubation, surviving cells were transferred in LB and allowed to grow overnight (37°C, 300 rpm).

Creation of the DNA libraries for Illumina sequencing

Genomic DNA from 4.10⁹ cells was extracted by phenol-chlorophorm and sheared by sonication to generate fragments from 200 to 400bp which were purified from a 2% agarose gel. Following end-repair and dA-Tailing (NEB#E6050S and NEB#E6053), sheared fragments were ligated to illumina adapters. Then, Illumina primers P5 and P7 were fused to the fragments carrying the transposon sequence by PCR. Primer P7 carries a specific index (6 bp sequence) allowing to identify the sample. Different P7-indexes were thus used for the preparation of each samples. The quality of the libraries (concentration and fragments' size) was verified by Qubit and Agilent dosage. All libraries were then pooled together at same concentration.

NGS and subsequent analysis

Sequencing was performed by the high-throughput sequencing core facility of I2BC (Centre de Recherche de Gif – <http://www.i2bc.paris-saclay.fr>) using an Illumina NextSeq500 instrument. Approximately 5 million single-end 75 bp reads per sample were uniquely aligned to the IHMA87 genome using Bowtie2 with default settings (Langmead and Salzberg, 2012). Read counts per feature were then obtained with htseq-count (Anders et al., 2015) using either coding or intergenic regions as features. Differential mutant abundance between plasma and

LB growth was assessed using DESeq2 (Love et al., 2014). For visualization, reads starting positions were extracted from alignment files to represent transposon insertion sites, which were then normalized to each sample reads number. Julian Trouillon, 3rd year PhD student of the team, performed the bioinformatic analysis.

Appendices

Participation to scientific communications

17th International Biennial Conference, Pseudomonas 2019 (Putrajaya, Malaysia)

Poster presentation

Award: 1st prize for the best student poster presentation

The role of complement in the fight against *P. aeruginosa* in human whole blood

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Pseudomonas aeruginosa is the third pathogen the most frequently isolated during nosocomial bacteremia. The interaction between the pathogen and the immune system in the circulation is not well understood, notably the component of the immune system, which is actively involved in the fight against *P. aeruginosa* in the blood, has not been identified yet. The goal of this study is to gain insights into the interactions between the different immune components present in the human blood and *P. aeruginosa* by using an *ex vivo* model. We found that *P. aeruginosa* strains expressing different virulence factors/toxins display variable capacity of survival in the human blood, which was independent of their cytotoxicity on cultured cells. Phagocytosis, Neutrophil Extracellular Traps and killing by the complement had variable contribution in bacterial elimination depending on the strain. The most resistant strains in the whole blood were able to escape the complement killing. Interestingly, elimination of the complement-sensitive strains followed the kinetics reminiscent of ciprofloxacin or tobramycin actions, where 99% of the bacterial population was rapidly eliminated within the first hour of incubation, but the killing rate slowed drastically, leaving a 0.1% surviving population able to persist without dividing even after 6 hours. This so-called “persister” phenotype was observed for all the complement-sensitive strains, and our preliminary data show that this phenomenon is observed in many other Gram-negative bacteria. These results demonstrate that although the complement is essential for killing of the majority of bacterial population in the blood, *P. aeruginosa* have developed two strategies to cope with the complement system; while some strains are complement-resistant, others are able to form persister cells. This may explain the success of *P. aeruginosa* in provoking bacteremia.

Deciphering mechanisms used by the pathogen *Pseudomonas aeruginosa* to face the innate immune system in human whole blood

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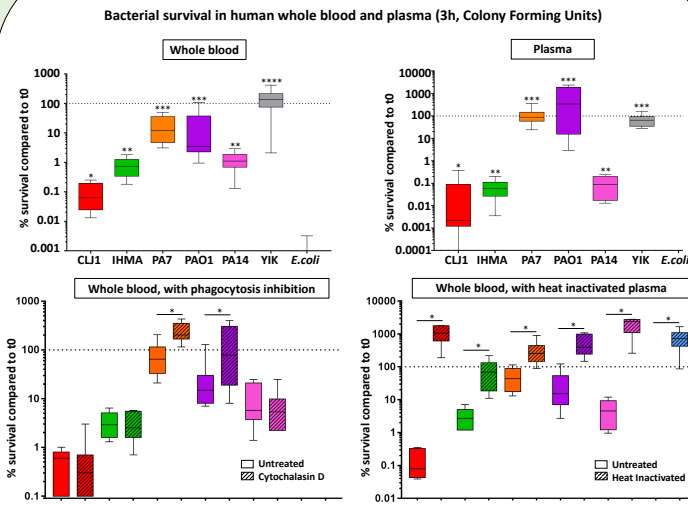
Context

Pseudomonas aeruginosa is a Gram negative bacteria which is one leading cause of nosocomial infections. Its main targets are immunocompromised and cystic fibrosis patients. While this pathogen usually induces localized infections, it can, in the worst cases, cross the endothelial barriers and join the circulation, causing life threatening bacteraemia. In blood, the bacteria faces the innate immune system, composed of phagocytic cells and the proteins of the complement system, which goal is to detect and eliminate foreign invaders. Even though the interactions between *P.aeruginosa* and the immune effectors have been studied *in vitro*, it is still unclear how the bacteria behaves when in contact with whole blood. We thus decided to use an *ex vivo* model of infection with human whole blood to answer three major questions:

- Do all *P.aeruginosa* strains have the same survival in human whole blood?
- What are the blood immune components actively engaged in the fight against *P.aeruginosa*?
- What are the mechanisms used by the bacteria to deal with these immune effectors?

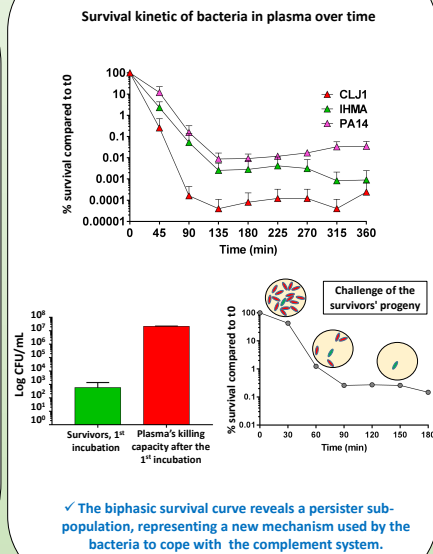
Bacteria used in this study		
Strains	Virulence factor	Origin
CLJ1		Haemorrhagic Pneumonia
IHMA	Exolysin A	Urinary Tract Infection
PA7		Burn
PAO1	T3SS ⁺ , ExoS	Wound
PA14		Burn
YIK	T3SS ⁺ , ExoU	Bacteraemia
<i>E.coli</i> K12	-	Laboratory

Survival in whole blood is driven by the ability of the strains to resist complement



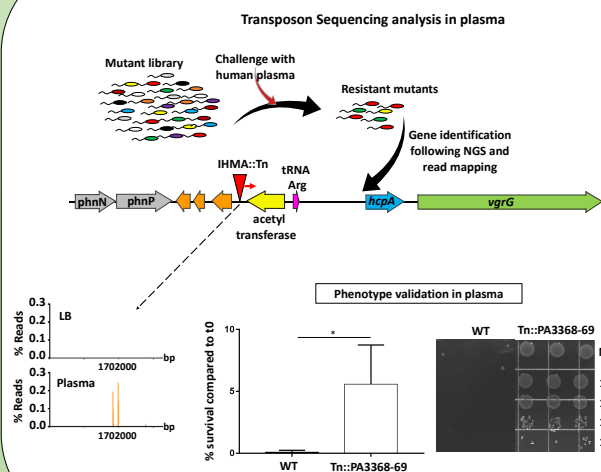
✓ In blood the complement system is essential for the fight against *P. aeruginosa*.

Complement-sensitive strains form persisters



✓ The biphasic survival curve reveals a persister sub-population, representing a new mechanism used by the bacteria to cope with the complement system.

Tn-seq identifies new genes involved in complement-bacteria interaction

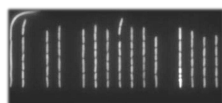


✓ 7 genes/pathways identified as affecting the survival of *P.aeruginosa* in plasma

Rank	Adjusted p-value	Gene	Function	Validation (increased survival compared to WT)
1	6,4.10 ⁻³³	mucD	Alginate synthesis	-
8	1,1.10 ⁻⁶	mucA		
10	2,9.10 ⁻⁴	mucB		
1	2,2.10 ⁻⁴	PA4032-MucE	Regulation of LPS O-antigen chain length	-
2	2,2.10 ⁻⁴	nadB-argU		
6	9,1.10 ⁻⁴	PA0759-PA0760		
9	3,2.10 ⁻²	PA0758-PA0759	Biotin synthesis	10x
2	5,8.10 ⁻¹⁰	himD		
7	1,7.10 ⁻³	wbpA-wzz		
8	1,4.10 ⁻²	wzz-himD	Type 4 pili biogenesis	-
3	6,7.10 ⁻⁹	bioF		
5	3,2.10 ⁻⁸	bioA		
6	4,2.10 ⁻⁸	bioC	Unknown function	1000x
7	3,4.10 ⁻⁷	bioB		
4	1,0.10 ⁻⁸	fppA		
14	3,1.10 ⁻³	PA4294	Purine synthesis	100x
4	2,9.10 ⁻⁴	pprB-tadG		
5	4,7.10 ⁻⁴	tadG-PA4298		
3	2,9.10 ⁻⁴	PA3368-PA3369	Outer membrane protein, unknown function	-
9	4,3.10 ⁻²	purN		
11	1,3.10 ⁻²	purF		
12	1,5.10 ⁻²	purL	-	-
16	9,4.10 ⁻²	purM		
17	1,8.10 ⁻²	purD		
13	2,0.10 ⁻³	opmQ		

What's next?

- ❖ Characterize the persister sub-population in human plasma, using the mother machine (microfluidic).
- ❖ Characterize the mutants identified by Tn-Seq
 - Interact less with the complement system?
 - Degrade these immune proteins?
 - Form more persisters?



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Mini congrès Pseudomonas France 2019 (Voiron, France)

Oral presentation

Rôle du complément dans la lutte contre *P. aeruginosa* dans le sang total humain et stratégies utilisées par la bactérie pour y faire face

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Pseudomonas aeruginosa (*P.a*) est une des causes majeures de bactériémie nosocomiale, infection sévère à mauvais pronostic pour le patient. Les interactions entre ce pathogène et le système immunitaire dans la circulation restent encore mal comprises, notamment, le composant du système immunitaire jouant un rôle prépondérant dans la lutte contre *P.a* dans le compartiment sanguin n'a pas encore été identifié. Mon projet vise donc à étudier les interactions entre *P.a* et les différents composants du système immunitaire présents dans le sang humain, en utilisant un modèle d'infection *ex vivo*. Nous avons observé que des souches de *P.a* exprimant différents facteurs de virulence/toxines présentent des capacités très variables à survivre dans le sang, et ce indépendamment de leur cytotoxicité sur les cellules immunitaires isolées. Nous avons également montré que le système du complément est essentiel à l'élimination de ce pathogène dans le sang, soit par opsonisation, soit par lyse des bactéries, et que les souches les plus résistantes dans le sang sont celles qui résistent à l'action lytique du complément. Enfin, nous avons observé que l'élimination des souches sensibles à la lyse par le complément suit une cinétique similaire à celle observée en présence d'antibiotiques tels que la ciprofloxacine ou la tobramycine. En effet, plus de 99% de la population bactérienne est rapidement détruite durant la première heure d'incubation en plasma, puis le taux d'élimination ralentit, laissant une population de survivants représentant moins de 0,1% de la population initiale, capable de persister sans se diviser même après 6 heures d'incubation. Ce phénotype "persister" est observé avec toutes les souches de *P.a* sensibles au complément, et également chez de nombreuses autres espèces de bactéries Gram⁻. Ces résultats montrent que ce pathogène a développé deux types de stratégies afin de survivre face au système du complément : soit par résistance, soit par acquisition d'un phénotype "persister". Cela pourrait expliquer pourquoi *P.a* est la troisième cause de bactériémie nosocomiale. Grâce à des expériences de Tn-Seq, nous avons également pu identifier des régions génomiques jouant un rôle important dans l'interaction entre le système du complément et *P.a*, le rôle précis de ces régions est en cours d'évaluation.

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Strategies employed by *Pseudomonas aeruginosa* to withstand the innate immune system in human whole blood

Pseudomonas aeruginosa (Pa) is a Gram-negative opportunistic pathogen primarily affecting immunocompromised and cystic fibrosis patients. In addition to lung and urinary tract infections, Pa is often isolated from blood of bacteremic patients. Blood stream infections caused by this particular pathogen register with higher mortality rates compared to septicemia associated with other bacteria. Once in the circulation, Pa faces the innate immune system of the blood, however the strategies employed by this pathogen to escape killing by the complement system and circulating phagocytes have not been investigated in whole blood. In this work, we assessed the survival of a collection of six Pa strains in human whole blood. We found that rather than cytotoxicity toward circulating leukocytes, complement resistance is the main driver of survival in the blood. The origin, serotype of the isolate, as well as the nature of the toxins expressed by the strains did not affect their survival. We also discovered that complement-sensitive strains prevent their eradication through the generation of a minor subpopulation of “evaders”, able to withstand complement-mediated lysis. Depending on the strain, between 0.0001 and 0.01% of the initial population survived in human plasma. The characterization of evaders showed that they harbor a transient persister-like phenotype, and are also observed in *Acinetobacter baumannii*, *Burkholderia multivorans*, enteroaggregative *Escherichia coli*, *Klebsiella pneumoniae*, and *Yersinia enterocolitica*. Formation of evaders likely represent an efficient strategy for complement-sensitive bacteria to persist within the blood and disseminate through the host. Using a genome-wide screen of a transposon mutant library in human plasma (Tn-Seq), we identified new bacterial factors influencing the interplay between Pa and the complement system. We observed that bacteria negatively affected in *de novo* biotin and purine synthesis survive better in plasma than the parental strain. In addition, Tn-Seq identified an operon of three genes, that we named “srg” for “serum resistant genes” whose products are predicted to be membrane bound. SrgABC overexpression confers tolerance to plasma (1000 fold-increased survival compared to wt) and triggers an overproduction of alginates, suggesting complex interplay between bacterial membrane, exopolysaccharides and complement-mediated lysis.

Stratégies employées par *Pseudomonas aeruginosa* afin de résister au système immunitaire inné dans le sang humain

Pseudomonas aeruginosa (Pa) est une bactérie à Gram négatif considérée comme pathogène opportuniste, principalement retrouvée chez les patients immunodéprimés ou atteints de mucoviscidose. Cette cause majeure d'infections nosocomiales est à l'origine de pathologies pulmonaires et urinaires, mais est également fréquemment isolée à partir du sang de patients atteints de bactériémies. Les infections sanguines causées par ce pathogène ont été montrées comme particulièrement mortelles par rapport aux septicémies associées à d'autres espèces bactériennes. Une fois entré dans la circulation, Pa doit faire face au système immunitaire inné du sang, cependant les stratégies employées par ce pathogène afin de résister au système du complément et aux cellules phagocytaires n'ont jamais été étudiées dans le sang total. Grâce à l'étude de la survie d'une collection de six souches de Pa dans le sang total humain, nous avons pu démontrer que plutôt que la cytotoxicité envers les leucocytes circulants, la résistance à la lyse par le complément est le principal moteur de la survie bactérienne. De plus, l'origine, le sérotype de l'isolat, ainsi que la nature des toxines exprimées par les différentes souches n'impactent pas leur survie dans cet environnement. Nous avons également découvert que les bactéries sensibles au complément étaient capables d'éviter une éradication totale, grâce à la formation d'une sous-population d'“evaders” résistante à l'action lytique du complément. Suivant la souche étudiée, cette population représente entre 0,0001 et 0,01% de la population initiale. Ces cellules rares présentent un phénotype transitoire proche des persisteurs, et ont également été observées chez *Acinetobacter baumannii*, *Burkholderia multivorans*, *Escherichia coli* entéro-agrégatif, *Klebsiella pneumoniae*, et *Yersinia enterocolitica*. La formation d'evaders représente probablement une stratégie efficace permettant aux souches sensibles au complément de persister dans le sang et ainsi disséminer dans l'organisme. Grâce au criblage d'une banque de mutants de transposition en plasma humain (Tn-Seq), nous avons pu identifier de nouveaux facteurs bactériens impliqués dans l'interaction entre Pa et le système du complément. Nous avons observé que des bactéries incapables de synthétiser leurs biotine et purines survivent mieux en plasma par rapport à la souche parentale. Également, le Tn-Seq nous a permis d'identifier un opéron de trois gènes, que nous avons nommé “srg” pour “serum resistant genes”, dont les produits sont prédits comme étant localisés à la membrane. La surexpression des protéines SrgABC confère à la bactérie une survie accrue en plasma (augmentation d'un facteur 1000 par rapport à la souche sauvage), et induit une surproduction des alginates chez cette dernière, suggérant l'existence d'une interaction complexe entre la membrane bactérienne, la sécrétion d'exopolysaccharides et l'action lytique du complément.