The Great Escape - Investigating Complement Evasion Mechanisms in Pathogenic Bacteria

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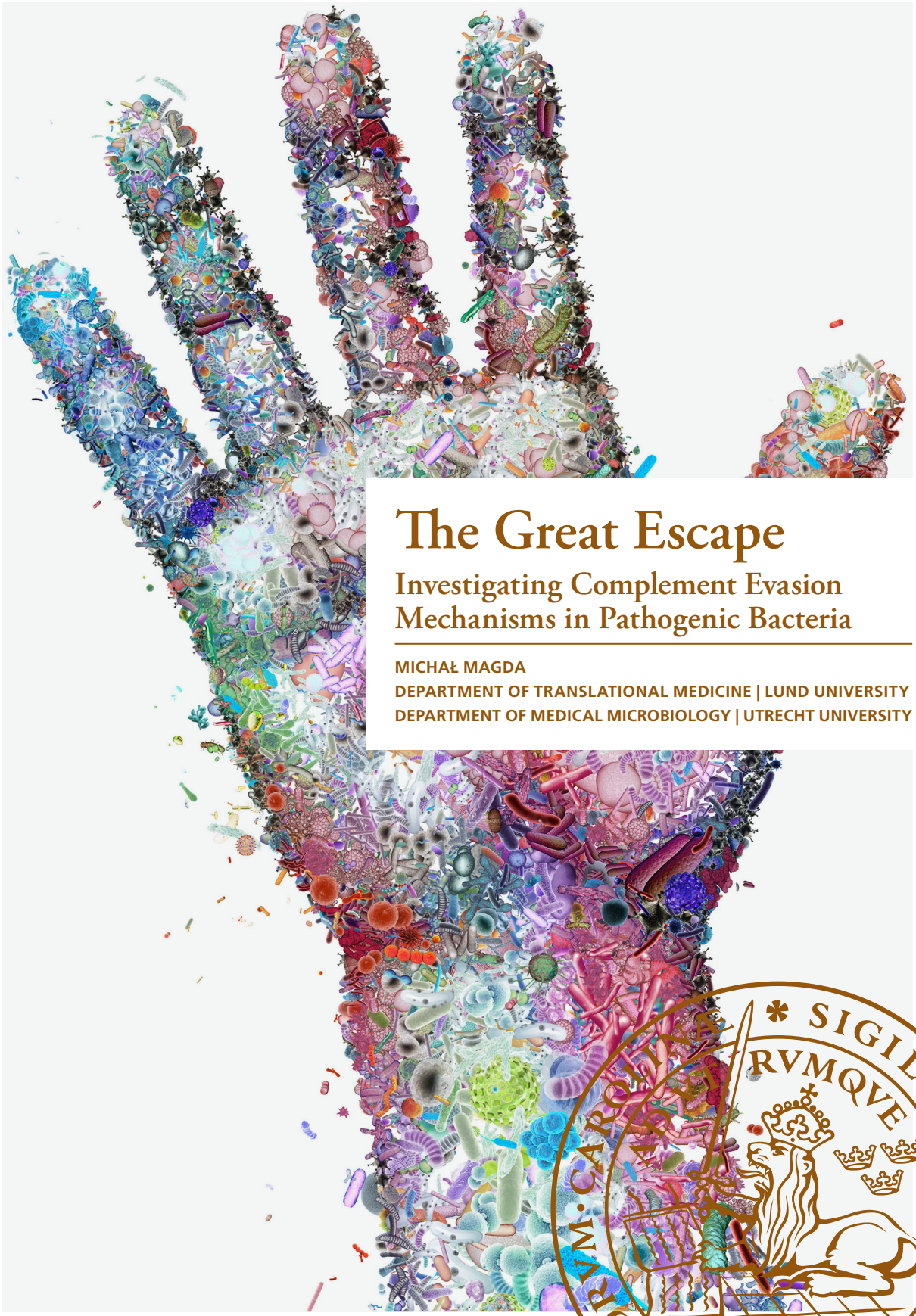

The Great Escape

Investigating Complement Evasion Mechanisms in Pathogenic Bacteria

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LUND
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Title and subtitle: The Great Escape. Investigating Complement Evasion Mechanisms in Pathogenic Bacteria.

Abstract: The complement system, a crucial part of the innate immune system, protects the human body during bacterial infections. Complement comprises three pathways, classical, lectin, and alternative, which activate as a protein cascade. Activation of these pathways leads to the opsonization of pathogens with C3 fragments for phagocytosis, chemotaxis of phagocytes to the infection site, and deposition of MAC to lyse the bacteria.

However, pathogens such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes* have evolved sophisticated mechanisms to evade complement-mediated killing. These pathogens are responsible for life-threatening nosocomial infections, causing diseases such as pneumonia, urinary tract infections, bloodstream infections, skin infections, and meningitis. They produce numerous virulence factors involved in host virulence, complement resistance, environmental persistence, and antibiotic resistance. In this project, we aimed to identify their complement evasion mechanisms.

In Papers I and II, we investigated the serum resistance of *Acinetobacter* spp. Bacteria were significantly serum-resistant despite activation of the complement system. Some *A. baumannii* isolates demonstrated substantially higher virulence in the *Galleria mellonella* animal model and exhibited much lower MAC deposition, indicating a complement evasion mechanism. Genome sequencing highlighted possible virulence mechanisms for further study. In Paper III, we analyzed the relationship between antibiotic and complement resistance in *K. pneumoniae*. Colistin-resistant isolates were more virulent against *G. mellonella* larvae and more prone to infect mice in the pneumonia model. They also presented significantly increased complement resistance. In paper IV, we evaluated the efficacy of the fusion proteins against *S. pyogenes* infection. The FH6-7/hFc protein bound and displaced serum-acquired FH from the bacterial surface, increasing the complement-mediated opsonization and phagocytosis of the bacterium. When used as a therapeutic, the protein significantly increased the survival rates of mice during the sepsis model.

This dissertation emphasizes the importance of further studies on human pathogens and their still unknown complement evasion strategies. A better understanding of bacterial evasion is essential for developing new treatment options. Exploiting bacterial evasion mechanisms using fusion proteins presents a promising approach to combat various bacterial infections and suggests a potential new direction in infectious disease therapy.

Keywords: complement system, human pathogens, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, virulence factors, antibiotic resistance, fusion protein

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Scio me nihil scire.

Preface

Dear Reader,

The book you are holding in your hands is the culmination of several years of effort. Together with my colleagues, we have worked tirelessly to expand our knowledge of the complement system and bacterial infections.

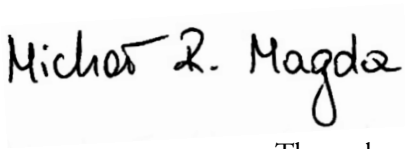
The bacteria we have studied are dangerous human pathogens of the utmost importance to the healthcare system. Therefore, I believe our research has been beneficial to society. The work was challenging and often frustrating but ultimately satisfying as it provided exciting insights.

The book itself was the most challenging part of my project. It took me countless hours of work and dedication, but I am delighted to have completed it. I sincerely hope you will read this book and learn something from it. I certainly learned a lot while writing it.

I have done my best to compile as much relevant information as possible about the complement system and the pathogenicity of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes*. However, these fields are vast and contain an immense amount of fascinating knowledge.

Therefore, this is not the best thesis in the world.

This is just a tribute.



The author
Malmö, June 2024

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Kristian, thank you for the collaboration. Thank you for helping with my project.

To my collaborators who contributed to the publications: **Maisem** and **Derek**, thank you for collecting the *Acinetobacter* isolates that started this project. **Thomas**, thank you for providing us with mutant strains and for your feedback. **Christian** and **Chaitanya**, thanks for the collaboration and help with genome sequencing of my isolates. **Sanjay**, thank you for providing us with fusion proteins. **Lisa**, thank you for your hard work on the GAS and fusion proteins project. **Oskar L**, **Oskar T**, and **Valdemar**, thank you for contributing to the *Klebsiella* manuscript. **Oleksandr** and **Dmytro**, thank you for collecting *Klebsiella* isolates for publication. **Wendy**, you did a great job during your internship. I am grateful for your contribution to the project and your patience with me as your supervisor.

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To all the current and former members of the **Medical Protein Chemistry** lab. To the students and postdocs that I have had the opportunity to work with over the past years. **Frida**, you are the heart and soul of this lab. We could not survive without you! **Ewelina**, thank you for more than a decade of friendship! Thank you for all the parties, trips, and hanging out after work. **Emre**, thank you for having lunch and fika with me more times than I can count. **Tomi**, thank you for playing basketball and partying together. Thanks for introducing me to baklava and moussaka! **David**, it was great working with you. I appreciate what you have taught me. I enjoyed your humor and discussions. **Alex**, we „wasted" so many hours talking about football, Sweden, and weird random topics. Thanks for being an awesome office buddy! **Klaudia** and both **Karolinas**, it was nice to work with fellow Polish students. **Eleni**, **Kasia**, and **Goutham**, thanks for the funny discussions and beers after work. **Gilar**, **Kostas**, **Ben**, and **Myriam**, thank you for creating a lovely atmosphere in the lab and for your feedback during meetings. **Damian**, thank you for the Formula 1 discussions and Sunday football league.

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Thank you to my **parents**, my **brother**, and **family** for supporting me during my PhD and for believing in me all my life!

Finally, to **Lau**, ¡Gracias... totales!

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List of papers included in this thesis.

Paper I

Magda M, Bettoni S, Laabei M, Fairley D, Russo TA, Riesbeck K, Blom AM (2022). Clinical Isolates of Acinetobacter spp. Are Highly Serum Resistant Despite Efficient Recognition by the Complement System. *Frontiers in Immunology, 13, 814193. Doi: 10.3389/fimmu.2022.814193*

Paper II

Magda M, Boschloo W, Bettoni S, Fairley D, Russo TA, Giske CG, Tellapragada C, Rooijakkers SHM, Riesbeck K, Blom AM (2024). Acinetobacter baumannii clinical isolates resist complement-mediated lysis by inhibiting the complement cascade and improperly depositing MAC.
(manuscript submitted to the Journal of Innate Immunity)

Paper III

Ljungquist O*, **Magda M***, Giske CG, Tellapragada C, Nazarchuk O, Dmytriiev D, Thofte O, Öhnström V, Matuschek E, Blom AM, Riesbeck K (2024). Pan-drug-resistant Klebsiella pneumoniae isolated from Ukrainian war victims remain hypervirulent.
(manuscript submitted to the Journal of Infection)
* Equal contribution

Paper IV

Blom AM*, **Magda M***, Kohl L*, Shaughnessy J, Lambris JD, Ram S, Ermert D (2017). Factor H-IgG Chimeric Proteins as a Therapeutic Approach against the Gram-Positive Bacterial Pathogen Streptococcus pyogenes. *Journal of Immunology 199(11), 3828–3839. Doi: 10.4049/jimmunol.1700426*
* Equal contribution

List of papers not included in this thesis.

Paper I

Jusko M, Miedziak B, Ermert D, **Magda M**, King BC, Bielecka E, Riesbeck K, Eick S, Potempa J, Blom AM (2016). FACIN, a Double-Edged Sword of the Emerging Periodontal Pathogen Filifactor alocis: A Metabolic Enzyme Moonlighting as a Complement Inhibitor. *Journal of Immunology 197(8), 3245–3259. Doi: 10.4049/jimmunol.1600739*

Paper II

Ermert D, Weckel A, **Magda M**, Mörgelin M, Shaughnessy J, Rice PA, Björck L, Ram S, Blom AM (2018). Human IgG Increases Virulence of Streptococcus pyogenes through Complement Evasion. *Journal of Immunology 200(10), 3495–3505. Doi: 10.4049/jimmunol.1800090*

Paper III

Bettoni S, Dziedzic M, Bierschenk D, Chrobak M, **Magda M**, Laabei M, King B, Riesbeck K, Blom AM (2024). C4b-Binding Protein and Factor H attenuate NLRP3 Inflammasome-mediated Signalling Response during Group A Streptococci Infection in Human Cells.
(manuscript submitted to the Journal of Innate Immunity)

Abbreviations

AMR	Antimicrobial Resistance
AP	Alternative Pathway
ATCC	American Type Culture Collection
C1-INH	C1 Inhibitor
C3aR	C3a Receptor
C4BP	C4 Binding Protein
C5aR1	C5a Receptor 1 (CD88)
C5aR2	C5a Receptor 2
CCP	Complement Control Protein
CD	Cluster of Differentiation
CKP	Classical *Klebsiella pneumoniae*
Clu	Clusterin
CP	Classical Pathway
CR1	Complement Receptor 1 (CD35)
CR2	Complement Receptor 2 (CD21)
CR3	Complement Receptor 3 (CD11b/CD18)
CR4	Complement Receptor 4 (CD11c/CD18)
ECM	Extracellular Matrix
FB	Factor B
FD	Factor D
FH	Factor H

FI	Factor I
GAS	Group A *Streptococcus*
HvKP	Hypervirulent *Klebsiella pneumoniae*
IFN	Interferon
IL	Interleukin
LOS	Lipooligosaccharide
LP	Lectin Pathway
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MASP1	MBL-Associated Serine Protease 1
MASP2	MBL-Associated Serine Protease 2
MBL	Mannose-Binding Lectin
MDR	Multidrug-resistant
NETs	Neutrophil Extracellular Traps
NFκB	Nuclear Factor-κB
OMP	Outer Membrane Protein
OMV	Outer Membrane Vesicle
PAMP	Pathogen-Associated Molecular Pattern
PDR	Pandrug-resistant
PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PLD	Phospholipase D
ROS	Reactive Oxygen Species
sMAC	Soluble Membrane Attack Complex
T6SS	Type VI Secretion System
TLR	Toll-like Receptor

TNFα	Tumor Necrosis Factor α
Vn	Vitronectin
WHO	World Health Organization
XDR	Extensively drug-resistant

Introduction

The human body is constantly exposed to bacteria. The human immune system, which consists of innate and adaptive systems, works tirelessly to protect us from bacterial infection. While the adaptive system matures over time, the innate system protects us from the beginning of our lives. The complement system plays a critical role in innate immunity. This intricate system of proteins and enzymes works with antibodies and professional phagocytic cells to eliminate pathogens.

While many microorganisms do not pose a serious threat, many pathogenic bacteria cause life-threatening infections. After entering the human body, bacteria multiply rapidly and spread swiftly throughout the organism. The complement system intervenes to prevent their spread. However, pathogens develop strategies to evade recognition and neutralization by complement. Science is developing novel therapeutics, such as antibiotics and vaccines, to support the immune system. Unfortunately, some pathogens can mutate and create new mechanisms faster than medicine can address them.

As a result, we are in a constant race against bacteria. Although scientists have identified many evasion mechanisms, many more are still to be discovered. Therefore, in this thesis, we studied the human pathogens *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes* with the aim of better understanding their evasion mechanisms, characterizing their behavior in the clinical environment, and proposing a potential treatment option that would overcome their virulence mechanism.

Complement system

History of the complement system

The discovery of the complement system was achieved by several pioneers in immunology. George Nuttall observed in 1888 that blood heated to 55 °C lost its ability to kill bacteria [1]. A similar observation was made by Hans Ernst August Buchner in 1891 [2]. He called this heat-labile fraction of blood alexin (to ward off in Greek). Shortly after, in 1895, Jules Bordet confirmed their results and presented the idea of a heat-stable blood component, which he referred to as a sensitizer (*substance sensibilisatrice* in French) [3]. However, immunology needed a few more years, and the influence of Paul Ehrlich was required to better understand the duality of blood's antibacterial properties. In 1899, he published results showing the presence of amboceptors (Bordet's sensitizers), heat-stable factors now known as antibodies [4]. He speculated that amboceptors had a bifunctional capacity to bind antigens on bacteria and alexin simultaneously. Therefore, he proposed a new name for alexin – complement – since complement, in his theory, supported the functions of amboceptors. His influence on immunology was appreciated in 1908 when he and Ilya Mechnikov (for his work on phagocytosis [5]) were awarded the Nobel Prize in Physiology or Medicine. Jules Bordet's research was also highly valued as he received his Nobel Prize in the same category in 1919.

Complement research in the early 20th century provided the foundations for the classical complement pathway. In 1907, Adolfo Ferrata and Erwin Brand discovered the C1 and C2 proteins [6, 7]. Studies of cobra venom and yeast, among others by Arthur Coca, led to the discovery of C3 [8, 9]. Similarly, treating human serum with ammonia revealed the existence of C4 [10]. Thanks to the development of the technology and the work of H. J. Müller-Eberhard and Ulf R. Nilsson, C4 was successfully purified in 1963 [11], followed quickly by the isolation of the other components C5 [12], C6 [13], C7 [13], C8 [14], and C9 [15].

The complement community accepted the classical complement pathway dependent on the antibody binding. The assumption of the existence of another pathway was suspected during the early experiments on the cobra venom factor. Still, the final discovery of the so-called properdin pathway in 1954 caused much controversy. Louis Pillemer's research led to the discovery of an alternative pathway whose activation is independent of antibodies [16]. The most prominent skeptic of Pillemer's studies was Robert Nelson, who published his observations in 1958, claiming that Pillemer had made mistakes in his experiments and that the activation was due to the antibody-antigen complex [17]. However, looking back at Nelson's observations, we can identify errors in his assumptions and experimental procedures [18, 19]. Faced with criticism from society, Pillemer took his own life in 1957 – new studies in the '60s and '70s confirmed Pillemer's concept.

The discovery of the lectin pathway is much less tragic and surprisingly recent. In 1978, Kawasaki et al. isolated the mannose-binding lectin (MBL) [20]. However, it was not until 1989 that answers to the functions of MBL were provided, with the studies on immunodeficiency in children [21, 22]. Later, in 1992, Matsushita and Fujita isolated the MBL-associated serine protease (MASP) and demonstrated that MBL from the lectin pathway activates complement similarly to the C1 complex from the classical pathway.

Currently, it is known that the complement system comprises over 50 different soluble and membrane-bound proteins and their fragments, including proenzymes, proteases, opsonins, anaphylatoxins, and inhibitors [23]. Although the initial studies on complement focused on its role in killing pathogens, it is now understood that complement and complement deficiencies are involved in many diseases, such as paroxysmal nocturnal hemoglobinuria (PNH) [24], atypical hemolytic uremic syndrome (aHUS) [25], or age-related macular degeneration (AMD) [26]. The discovery of complement-related diseases has presented a demand for novel treatment options. In 2007, the U.S. Food and Drug Administration (FDA) approved Eculizumab, a humanized monoclonal anti-C5 antibody, for treating hemoglobinuria [27, 28]. Eculizumab, the first complement drug sold under the name Soliris®, successfully blocks the cleavage of C5 and prevents activation beyond that point in the complement cascade. The clinical success of Eculizumab has led to the development of other similar drugs. Numerous antibodies, peptides, and proteins have been approved or tested in clinical trials against complement-mediated diseases [29].

Source of complement proteins

Complement was long thought to be mainly produced in the liver by hepatocytes. Most circulating serum proteins are *de facto* produced in hepatocytes, with C3 being the central molecule [30, 31]. C3 expression was demonstrated when studying individuals undergoing liver transplantations [32]. Nevertheless, there was no conclusive evidence of hepatocyte production for some complement proteins such as C1q, Factor D (FD), and properdin. Interestingly, the liver produces other C1 complex components, such as C1r and C1s, but not C1q. C1q was shown to be secreted by monocytes, macrophages, dendritic cells, endothelial cells, and epithelial cells. Also, in the brain, C1q can be produced by astrocytes and microglia [33]. Adipocytes are the leading producers of FD. However, as C1q, FD can also be produced by leukocytes and astrocytes. Properdin's sources are leukocytes and T cells [34]. Besides those produced in the liver, complement proteins can be secreted locally by fibroblasts, epithelial cells, endothelial cells, and leukocytes, with monocytes and macrophages as the leading complement sources [35]. Furthermore, various cells produce complement inhibitors and receptors, including monocytes, macrophages, dendritic cells, and lymphocytes. The local expression of complement can be constitutive but is often stimulated by cytokines [31, 34]. In general, the local secretion of complement molecules seems to play a role in complement regulation before systemic activation occurs.

Activation of the complement cascade

The complement system can be activated by any of its pathways: classical, lectin, or alternative (**Figure 1**). Initiation of the classical and lectin pathways requires the stimulation of specialized molecules such as C1q or MBL, whereas the alternative pathway is continuously activated by spontaneous hydrolysis. These initiating molecules activate the complement cascade by binding to antibodies (IgG, IgM) or by directly binding to pathogen-associated molecular patterns (PAMPs) on the surface of bacteria and to damage-associated molecular patterns (DAMPs) present in the molecules released from dying cells and tissues. This mechanism allows for the tight control of the activation of the complement system [36].

Classical pathway

The classical pathway (CP) is activated by the C1 complex, which is composed of one C1q protein and two serine proteases, C1r and C1s, in a ratio of 1:2:2. The complex has a molecular weight of 760-790 kDa and is stabilized by Ca^{2+} [37]. Activation of the complex occurs when C1q binds to the Fc regions of IgG and IgM antibodies. A single pentameric IgM structure is sufficient to activate C1q, while IgG oligomerization is required [38, 39]. However, C1q can be activated by means other than antibodies, including C-reactive protein (CRP), serum amyloid P component (SAP), and pentraxin-3 (PTX3). Similarly, the CP can be triggered by interaction with either apoptotic cells both directly [40] or via Annexins A2 and A5 [41], or bacterial cells through lipopolysaccharide (LPS) [42] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [43], which triggers the CP.

During activation, and in the presence of Ca^{2+}, C1q undergoes a conformational change that activates protease C1r. In consequence, C1r cleaves and activates the C1s [44], which then cleaves the C4 protein into C4a and C4b. The function of C4a is still unclear [45]. C4b binds to the surface near the C1 complex via the exposed thioester bond. Then, C2 is coupled with C4b in the presence of Mg^{2+}. Subsequently, C1s cleaves C2 into C2a, which has an unknown activity, and C2b. Together with C4b, C2b forms the CP C3 convertase C4b2b [23]. It is estimated that a single C1 complex may cleave up to 35 C4 and only 4 C2 molecules [46]. The formed C3 convertase cleaves the C3 protein into anaphylatoxin C3a and C3b. The C3b fragment, with its thioester bond exposed, binds to the surface close to the C3 convertase (within 100 nm) due to its short half-life of approximately 60 μs [47-49]. The newly formed C3b binds to the surface, acting as an opsonin, or binds to the pre-existing CP C3 convertase to form CP C5 convertase C4b2bC3b [50]. The resulting C5 convertase shifts its focus from C3 to C5 with an almost 1000-fold increase in affinity, cleaving C5 into C5a and C5b [51]. C5a is another anaphylatoxin, while C5b is required to initiate the complement system's terminal pathway (TP).

Lectin pathway

The lectin pathway (LP) is activated upon detection of carbohydrates commonly found on pathogens' surfaces, such as mannose, glucose, fucose, galactose, N-acetyl-D-glucosamine (GlcNAc), or N-acetyl-D-galactosamine (GalNAc). LP's recognition molecules are MBL, collectins (collectin-10 (CL-10), collectin-11 (CL-11)), and

ficolins (ficolin-1 (M-ficolin), ficolin-2 (L-ficolin), ficolin-3 (H-ficolin)). LP requires the presence of a Ca^{2+} to bind and activate [52]. Correspondingly to CP, the serine proteases MASP-1 and MASP-2 activate LP by associating with MBL on the surface. Tight complexes need to be created since MASP-1 is required to activate MASP-2. Both proteases cleave C2, but only MASP-2 can cleave C4. Noteworthy, MASP-3 protein does not seem to play an essential role in LP activation but appears crucial for the activation of Factor D in AP [52]. After cleaving C4 and C2, the activation of the LP continues similarly to the CP until C5 cleavage occurs.

Alternative pathway

Interestingly, the alternative pathway (AP) does not need a recognition molecule as it is continuously activated at a low level. Such spontaneous activation was highly beneficial for primitive organisms that expressed C3 but lacked proper CP and LP [53]. The tick-over process, described by Nicol and Lachmann [54], involves the hydrolysis of the thioester bond of C3 to form $C3(H_2O)$, an active state molecule similar to C3b. $C3(H_2O)$ then binds Factor B (FB), which is then cleaved by serine protease Factor D (FD), releasing the Ba fragment and forming fluid-phase C3 convertase $C3(H_2O)Bb$ [55]. This complex is much more stable than $C3(H_2O)$ and more resistant to the inactivation by AP inhibitors – Factors H (FH) and I (FI). Subsequently, $C3(H_2O)Bb$ cleaves normal C3 into C3b, which can bind to the surface via its thioester bond and form a surface-attached convertase. The cleavage must occur within 100 nm of the available surface to enable C3b-binding. Otherwise, it will react with water again, creating more $C3(H_2O)$ [56]. The C3b-binding is primarily a random process, so pathogen and host cells can be opsonized. Fortunately, the C3b on host cells is quickly inactivated by FH, FI, and membrane-bound inhibitors [57].

Once the C3b is attached to the surface, in the presence of Mg^{2+}, it binds FB, which is then cleaved by FD. Interestingly, LP-associated MASP-3 can activate the proenzyme form of FD, activating AP [58]. The AP C3 convertase, C3bBb, is short-lived (~90 s). Therefore, properdin attaches to it, increasing its longevity several-fold [59]. The stabilized AP C3 convertase can further cleave C3 proteins, creating active C3b molecules capable of creating new C3 convertases, generating more C3b. The process supported by CP and LP C3 convertases is known as the amplification loop and is considered the foundation for 80-90% of the complement activity [60]. However, when the newly cleaved C3b binds to the AP C3 convertase, the complex changes its

formation to C5 convertase C3bBbC3b, altering its affinity to C5. Further cleavage of C5 generates C5b, which is necessary for TP activation.

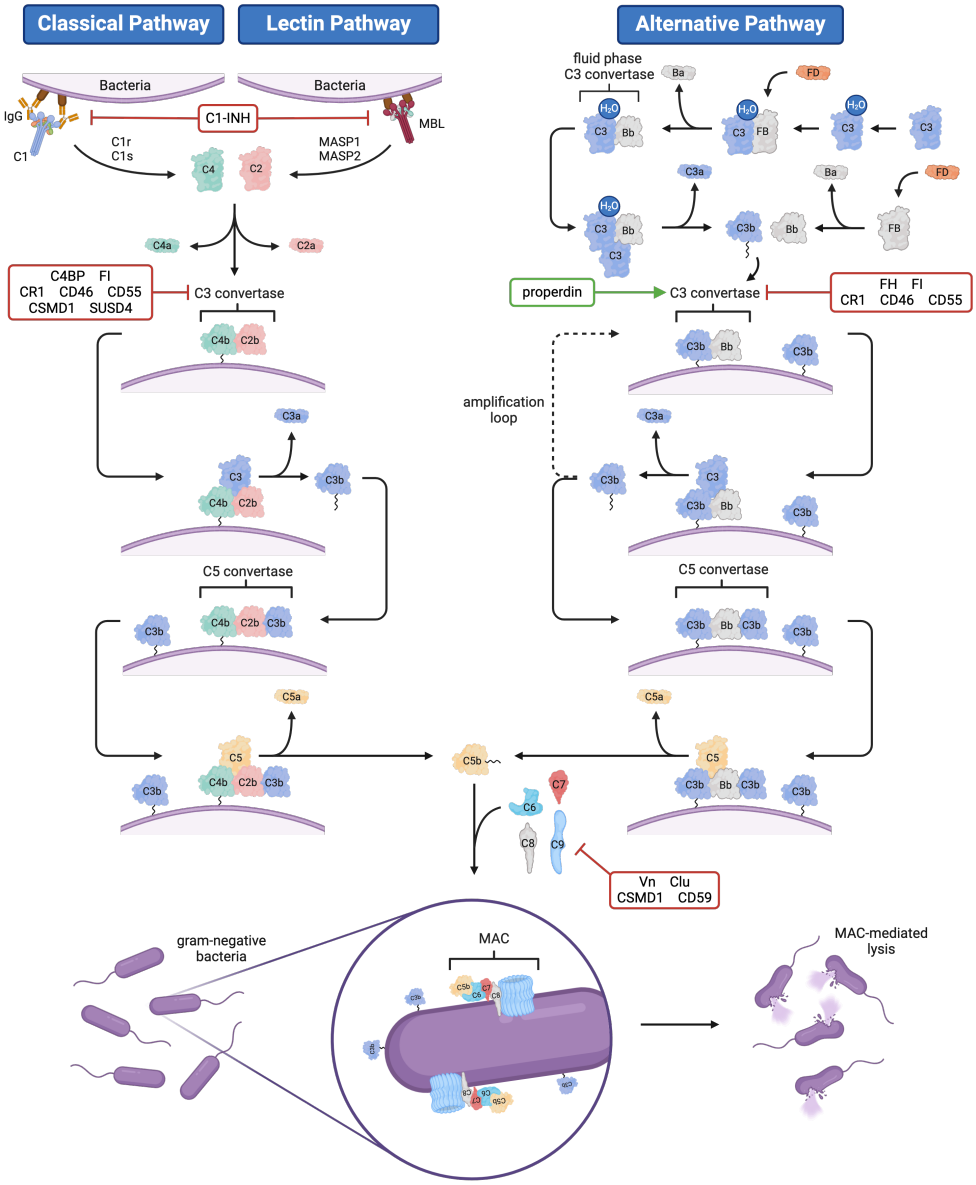

Figure 1. Simplified overview of the activation of the complement system on the surface of gram-negative bacteria.

Terminal pathway

The terminal pathway (TP) is the final step in activating the complement cascade. All pathways lead to the formation of the C5 convertase and cleavage of the C5 protein. The C5b fragment is the first protein involved in forming the membrane attack complex (MAC). C5b undergoes a conformational change that exposes the thioester bond, which binds to the C6 protein. Subsequently, C5b-6 binds to C7, and the resulting complex C5b-7 couples with the membrane. The fluid-phase C5b-7 complex can be inactivated by soluble inhibitors clusterin and vitronectin or can degrade spontaneously [61]. Once linked to the surface, C5b-7 binds to C8, which is then inserted into the membrane. Lastly, the C5b-8 complex recruits C9 proteins to form the complete MAC. Typically, 12-18 C9 proteins form a pore. The formation of MAC can be inhibited at this stage by membrane-bound inhibitor CD59, which binds to C8 and C9, preventing the unfurling of C9 required to create lytic pores [62]. Once enough of C9 has been polymerized, the MAC is completed. MAC deposits into the outer membrane of cells, forming a large pore that allows an influx of ions into the cell and, consequently, the cell's death.

Regulation of the system

The protein cascade must be strictly controlled to avoid damage to host cells caused by unwanted complement activation. Each step of the cascade has a dedicated inhibitor (**Figure 1**). The regulators of complement activation are either fluid-phase, circulating in the serum, or membrane-attached, expressed on the surface of host cells waiting for the deposition of complement components, which they can inactivate [63]. Each regulator comprises around 60 amino acid motifs called short consensus repeats (SCR), complement control proteins (CCP), or sushi domains [64].

Interestingly, properdin is the only canonical positive regulator of complement that has been described.

Fluid-phase inhibitors

C1-inhibitor (C1-INH) is a soluble, about 110-kDa serine protease inhibitor that blocks the activation of CP and LP. It is mainly produced by hepatocytes and circulates in plasma at a concentration of approximately 200 µg/ml. C1-INH belongs to the serpin family, which irreversibly binds to C1r and C1s, dissociating them from the C1 complex. Additionally, it inactivates MASP-1 and MASP-2 of the LP. Moreover, C1-INH regulates coagulation by binding to XIa, XIIa, and plasmin [65]. Deficiency in C1-INH can result in conditions such as hereditary angioedema, which is caused by uncontrolled activation of C1 and kallikrein. Cinryze™, C1-INH derived from human plasma, was approved by the FDA in 2008 for use against hereditary angioedema symptoms [66].

MAP-1 (Map44; 44 kDa) and MAP-2 (Map19; 19 kDa) are splicing variants of *MASP-1* and *MASP-1* genes, respectively. They belong to the MASP family but do not possess enzymatic properties. Thus, they are believed to regulate LP by competitively binding to MBL [67].

Factor I (FI) is one of the most important soluble inhibitors as it inactivates all pathways by cleaving the α-chains of C3b and C4b. FI cleaves C3b to iC3b, C3c, and C3dg, and C4b to C4c and C4d. FI's cleavage requires co-factors: soluble Factor H and C4BP and membrane-attached MCP (CD46) and CR1 (CD35). FI is an 88 kDa (two chains: 50 and 38 kDa) serine protease secreted mainly by hepatocytes. It is present in plasma with an average concentration of 34 µg/ml [65].

C4-binding protein (C4BP) is a major fluid-phase inhibitor of CP and LP. It interacts with C4b and detaches C2b, thus controlling the C3 convertase. It also serves as a co-factor for FI to cleave C4b permanently [63]. Interestingly, C4BP can also cooperate with FI to cleave C3b regulating AP. It is usually composed of seven α-chains and one β-chain (although isoforms without β-chains exist) with a total size of 570 kDa. C4BP interacts with C4b and C3b via CCPs 1-4 of its α-chains. The standard form circulating in plasma consists of protein S bound to the β-chain. Protein S helps C4BP bind to apoptotic and necrotic cells, thereby dampening the over-activation of complement. C4BP is present in plasma at a 200 µg/ml concentration and is primarily produced in the liver. Remarkably, there are no reports of complete C4BP deficiencies in humans [68].

Factor H (FH) serves as a negative regulator of AP. It competes with FB to bind C3b, accelerating the decay of the C3 convertase. It also acts as a co-factor to FI, which claves C3b into iC3b. The co-factor activity is significant since the binding of C3b by FH increases the affinity of FI towards C3b by 15-fold [65]. FH controls the tick-over process on host cell surfaces. It recognizes surface markers such as sialic acid, heparin, or glycosaminoglycans and binds to C3b deposited on the surface. Mutations and deficiencies in FH can lead to diseases such as aHUS, AMD, IgA nephropathy, or membranoproliferative glomerulonephritis. FH is a 155 kDa protein composed of 20 CCPs and circulates in serum at approximately 500 µg/ml. The first 4 CCPs harbor the co-factor activity and binding to C3b. Domains 7, 9-15, and 19-20 bind to the surface. FH is primarily produced in the liver but can also be made locally by various cells, such as epithelial and endothelial cells, monocytes, and platelets [69].

The FH protein family includes six additional FH-analogous proteins: Factor H-like protein 1 (FHL-1) and five Factor H-related proteins (FHR-1-5). FHL-1 is an alternative splice variant of FH, while FH-related proteins are produced by their own *CFR* genes. FH and FH-related proteins differ in structure and, thus, in their function. FHL-1, a non-glycosylated protein, contains CCPs 1-7 of FH and has a size of 49 kDa. FHL-1 exhibits inhibitory functions similar to FH but lacks CCPs 19-20, which prevents it from distinguishing the host surface. FHR proteins vary in size and structure, possessing five to nine CCPs. Their functions are not fully understood, as several studies have reported both inhibitory and activating effects on the AP simultaneously [64].

Clusterin (Clu, ApoJ) regulates MAC formation. Clu interacts with C7, C8β, and C9, as well as pre-formed MAC, due to the conformational changes that expose binding sites for Clusterin. The mature Clu is a glycosylated, heterodimeric protein consisting of α- and β-chains connected antiparallel by five disulfide bridges, with a molecular weight of 75-80 kDa. Clu is expressed in almost every tissue and is present in plasma ranging from 150-540 µg/ml [70].

Vitronectin (Vn, S protein) functions similarly to Clu. It interacts with the C5b-7 complex associated with the membrane, blocking its insertion into the membrane and the recruitment of C8 and C9 proteins. Additionally, Vn binds soluble complexes (sC5b-7, sC5b-8, sC5b-9), rendering them non-hemolytic and facilitating their removal. Vn circulates in the blood as a single-chain 75 kDa protein or a dimer composed of two chains, 65 kDa and 10 kDa. Vn is found in plasma as a monomeric protein; nonetheless, its activated form creates multimers ranging from 3- to 16-mers.

The serum concentration of Vn varies between 200-400 µg/ml, predominantly as a monomer. Vn is primarily produced in the liver but is also found in the brain, lungs, bronchoalveolar lavage fluid, smooth and skeletal muscles, kidneys, retina, and uterus [71].

Carboxypeptidase N (CPN) hinders the activity of anaphylatoxins C3a and C5a. CPN is a zinc metalloprotease that cleaves C-terminal arginines of anaphylatoxins, reducing their activity by 10-100-fold. C3a-desArg loses its activity completely, while C5a-desArg has a reduced ability to interact with receptors. Consequently, they no longer trigger the receptor-mediated signaling pathway. CPN comprises two catalytic and two regulatory subunits, forming a 280 kDa tetramer. The liver produces the enzyme, which is present in the blood at approximately 30 µg/ml [72].

Membrane-bound inhibitors

Complement receptor 1 (CR1, CD35) interacts with MBL, C1q, C4b, C3b, and iC3b with lower affinity. CR1 has a dual role as a complement receptor and inhibitor. As an inhibitor, it serves as the co-factor for FI during the cleavage of C4b and C3b, regulating all pathways. CR1 is a 200 kDa transmembrane glycoprotein whose extracellular domain comprises 30 CCPs. CCPs 1-3 possess C4b-binding and decay-accelerating activity. CCPs 8-10 and 15-17 have C3b/C4b-binding and co-factor activity, while CCPs 22-28 have C1q/MBL-binding activity. CR1 is expressed on B- and T-cells, follicular dendritic cells, monocytes, macrophages, erythrocytes, and glomerular podocytes. Although CR1 is mainly a transmembrane protein, a soluble version consisting only of an extracellular region is also present in serum, albeit at a very low level [73].

Membrane cofactor protein (MCP, CD46) functions as an FI co-factor similar to CR1. However, it does not possess decay-accelerating activity. CD46 is expressed on nearly all cell types but intriguingly not on erythrocytes. It is worth mentioning that, due to the alternative splicing, it is expressed mainly as a set of four isoforms ranging in size from 50-70 kDa. The extracellular domain of CD46 comprises four CCPs, with CCPs 2-4 responsible for binding C3b/C4b [74].

The decay accelerating factor (DAF, CD55) does not possess co-factor activity compared to CD46. Instead, it directly accelerates the decay of C3 convertase by dissociating C2b and Bb. The extracellular region of CD55 is composed of four CCPs, with CCPs 2-3 responsible for inhibiting CP and 2-4 inhibiting AP. Moreover, CD55

contains a glycophosphatidylinositol (GPI) anchor that attaches the protein to the cell surface. The predicted size of CD55 is around 70 kDa, but it may vary between 50-100 kDa depending on the cell type. CD55 is expressed on the surface of erythrocytes, leukocytes, NK cells, and epithelial cells. Interestingly, the soluble form of CD55 has been found in various body fluids such as plasma, tears, saliva, urine, synovial fluid, and cerebrospinal fluid [75].

Protectin (CD59) is a unique and crucial inhibitor of the MAC. It binds to the C5b-8 complex and restricts the binding, unfurling, and polymerization of C9. Its structure differs from that of CD46 and CD55, but CD59 is GPI-anchored to the surface like CD55. CD59 is a small, 18-25 kDa protein, expressed on all circulating cells, endothelial cells, and most epithelial cells, as well as on neurons, microglia, oligodendrocytes, and astrocytes. On average, a single erythrocyte contains 25-50 thousand CD59 molecules. Mutations in GPI production can lead to the absence of CD55 and CD59 on the cell surface, which can trigger paroxysmal nocturnal hemoglobinuria due to the vulnerability of erythrocytes to complement-mediated lysis [75, 76].

Sushi domain-containing 4 (SUSD-4) is a member of the SUSD family comprising six proteins. SUSD-4 has a size of 49 kDa and contains four CCPs and one transmembrane domain resembling CD46. It inhibits the deposition of C4b and C3b via CP and LP. It can also bind free C1q and in the C1 complex, blocking C2 cleavage and C3 convertase formation. Interestingly, it can also inhibit the AP, but the exact mechanism is unknown. SUSD-4 is expressed in the brain, particularly in neurons and oligodendrocytes [77].

CUB and Sushi multiple domains 1 (CSMD1) is a human inhibitor composed of repeated 14 N-terminal CUB-Sushi/CCP domains followed by 15 CCPs, totaling 390 kDa. CSMD-1 inhibits the C3 convertase, acts as a co-factor for FI to degrade C3b and C4b, and blocks the MAC formation by preventing the deposition of C7. It is produced in the testes, cerebral cortex, brain white matter, lungs, colon, breasts, and pancreas [77, 78].

Fluid-phase regulator

Properdin is the sole positive regulator of AP and complement system. Physiologically, the AP C3 convertase remains stable for approximately 90 seconds, but properdin can extend that time 5-10-fold. The protein exhibits the highest binding affinity towards

the C3bBb, followed by C3bB, C3b, and iC3b. Typically, it circulates in the multimeric form, where the activity of tetrameric properdin is greater than trimeric and greater than dimeric forms. It is a 53 kDa (monomer) glycoprotein found in plasma at 4-25 μg/ml. Unlike most complement proteins, properdin is primarily produced by leukocytes, and its deficiency increases the probability of meningococcal infection. Although the regulator's role in stabilizing the C3 convertase is well established, the protein's ability to activate AP is still debatable [79].

Primary functions of the complement system

Regarding host immunity and pathogen eradication, there are three primary outcomes of the complement system activation: 1) opsonization for phagocytosis, 2) recruitment and activation of phagocytes, and 3) deposition of MAC and cell lysis (**Figure 2**).

Opsonization

Activation of the complement system leads to the deposition of C3 fragments, predominantly C3b and iC3b, on the surface of bacterial cells. These opsonins are then recognized by professional phagocytic cells such as neutrophils, monocytes, macrophages, or dendritic cells, which leads to the adhesion to pathogens and subsequent phagocytosis. This is the most effective for the complement-mediated pathogen elimination process since it opsonizes both gram-positive and gram-negative bacteria [80]. A set of molecule-specific receptors recognizes opsonization.

As previously mentioned, CR1 may act as an inhibitor, but its primary function in phagocytosis is to recognize C3b and iC3b, with a slightly lower affinity towards the latter. It can also bind C1q, MBL, and C4b. CR1 is composed of 30 CCPs grouped into binding domains for different molecules. On phagocytes, CR1-mediated phagocytosis occurs in cooperation with Fc receptors, where CR1 binds C3b, and the Fc receptor binds Fc domains of deposited IgG [81].

Complement receptors 3 (CR3, CD11b/CD18) and 4 (CR4, CD11c/CD18) belong to the β_2-integrin family. They have similar β-subunits but differ in α-subunits. They have sizes of around 270 and 250 kDa, respectively. These receptors are involved in the adhesion process by interacting with intercellular adhesion molecules 1 and 2 (ICAM-1 and -2) and vascular cell adhesion molecule 1 (VCAM-1). During phagocytosis, both

receptors interact with iC3b. To interact, they require stimuli, such as inflammatory cytokines, to switch from an inactive bent to an active extended state. Moreover, their expression on phagocytes increases upon activation. Although CR3 and CR4 share many similarities, their iC3b binding sites and functions differ. CR3 appears to be a major phagocytic receptor, while CR4 is more involved in adhesion [81].

Complement receptors of the immunoglobulin family (CRIg) are present in a subpopulation of macrophages, mainly Kupffer cells in the liver. Their primary function is to remove bacteria from circulation. This is achieved by interacting directly with bacterial surfaces or the deposited C3b, iC3b, and C3c. The liver captures more than 90 % of circulating bacteria, making receptors on Kupffer cells critical in pathogen clearance [81].

Although antibody deposition is not a direct result of the complement-mediated process, it is worth mentioning that opsonization with IgGs is an essential step in phagocytosis. Surface-bound IgGs trigger the CP via the C1 complex and interact with Fc gamma receptors (FcγRs) in phagocytes. There are three types of receptors: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), with FcγRI having the strongest affinity for IgGs. Receptors have immunoglobulin-like domains responsible for binding to the Fc region of antibodies and an immunoreceptor tyrosine-based activation motif (ITAM) involved in the downstream signaling. Noteworthy, FcγRII has two isoforms, a and b, with FcγRIIb containing an immunoreceptor tyrosine-based inhibition motif (ITIM) that functions as an inhibitor of phagocytosis [82].

FcγRs and CRs work together to enhance each other's phagocytic functions. Both receptors facilitate the uptake of pathogens into cells. However, the mechanism of uptake differs depending on the receptor. CRs activate the actin and microtubule cytoskeletons, forming a phagocytic cup, so the opsonized bacteria sink into the cell. FcγRs activation leads to the formation of pseudopodia that pull bacteria inside. The binding of Fc to the receptors leads to receptor clustering and ITAM phosphorylation. This, in turn, recruits Syk kinase, which activates additional pathways and proteins such as phospholipase C (PLC), protein kinase C (PKC), and extracellular signal-regulated kinase (ERK). These pathways regulate the actin polymerization and pseudopodia function [82, 83].

The phagocytosis process becomes crucial after the pathogen is taken into the cells. The formation of the phagocytic cup and pseudopodia alters the membrane composition, creating a vesicle that contains the pathogen. The vesicle then fuses with early

endosomes, forming an early phagosome. This complex is characterized by Rab5, which recruits early endosome antigen 1 (EEA1). As the phagosome matures into a late phagosome, EEA1 recruits Rab7 into the membrane, allowing the fusion with late endosomes. Simultaneously, V-ATPase molecules accumulate, leading to phagosome acidification (pH 5.5 – 6.0). Late endosomes also provide lysosomal-associated membrane proteins (LAMPs) and proteases (cathepsins and hydrolases). Subsequently, the phagosome fuses with a lysosome to form a phagolysosome, which contains several mechanisms for destroying pathogens. Collectively, a very acidic pH, NADPH-oxidase-mediated production of reactive oxygen species (ROS), and several hydrolyzing enzymes (cathepsins, proteases, lysozymes, and lipases) contribute to pathogen elimination [81-83].

Complement mediated chemotaxis

Complement activation results in conformational changes in C2, C3, C4, and C5 proteins. Enzymatic cleavage of these proteins separates them into larger and smaller fragments. The larger fragments, such as C3b and C5b, bind tightly to form convertases, while the smaller fragments, such as C3a and C5a, are released into the fluid phase. The functions of C2a and C4a fragments in chemotaxis are still under debate. However, C3a and C5a are very well-known anaphylatoxins. It is noteworthy that C3a is a much less potent activator than C5a. However, their activity is significantly reduced after the cleavage of CPN, resulting in C3a-desArg and C5a-desArg. C3a and C5a are tiny peptides, approximately 10 kDa, that interact with their specific receptors, C3aR and C5aR [80].

C3aR is a G protein-coupled receptor expressed on the surface of neutrophils, monocytes, macrophages, basophils, eosinophils, mast cells, microglia, astrocytes, and neurons. It has a molecular weight of approximately 54 kDa. Binding of C3a stimulates downstream signaling pathways, including the activation of PLC, PKC, phosphoinositide 3-kinase γ (PI3Kγ), phosphokinase B (Akt), and ERK [80]. C3aR activation induces chemotaxis of eosinophils, mast cells, and macrophages. In macrophages, C3a stimulates the release of pro-inflammatory cytokines such as interleukins IL-1β and IL-6 and tumor necrosis factor α (TNFα). However, for neutrophils, it has the opposite effect, inhibiting movement and degranulation. Interestingly, C3a-desArg is unable to bind to its receptor [81].

C5aR1, similar to C3aR, is a G protein-coupled receptor expressed on various leukocytes and cell types. However, C5a-mediated activation of C5aR1 is a much more potent pro-inflammatory mechanism than C3a-C3aR. Unlike C3a, C5a-desArg can bind C5aR1 but has 90% lower activity than C5a [80]. The molecular size of C5aR1 is approximately 40-kDa. Activation of C5aR triggers downstream activation of several pathways, including phosphatidylinositol 3-kinase (PI-3K), protein kinase B (Akt), phospholipase D (PLD), PKC, and mitogen-activated protein kinase (MAPK). C5a attracts macrophages, neutrophils, basophils, monocytes, and mast cells, causing leukocyte degranulation and histamine release. Its proper functioning is essential for pathogen elimination, as C5aR is a potent activator of chemotaxis, adhesion, degranulation, and phagocytosis [81, 84, 85].

C5aR2, or C5L2, is a 36-kDa receptor related to C3aR and C5aR. It interacts with C5a and is therefore called a second C5a receptor. Interestingly, C5aR2 cannot be coupled with G protein and, as a result, cannot trigger downstream signaling pathways. C5aR2 is expressed on the surface of the same set of cells as C5aR1. Nevertheless, C5aR2 is mostly present intracellularly, which raises questions about its functions [86]. One suggestion is that C5aR2 acts as a decoy receptor by binding C5a and C5a-desArg to limit their interactions with C5aR1, thus limiting the inflammatory response. However, despite lacking a G protein, C5aR2 was found to interact with β-arrestin. C5aR2 may inhibit C5a-mediated signaling by interacting with β-arrestin or even modulate C5aR1 through direct interaction and heterodimerization. On the other hand, interaction with β-arrestin implies signaling that is independent of the G protein. Studies have presented evidence of ERK signaling upon C5aR2 and β-arrestin interaction stimulation. Therefore, the pro- and anti-inflammatory functions of C5aR2 may depend on the cell type and severity of the infection [81, 85, 86].

C3a and C5a are pro-inflammatory leukocyte stimulants. However, as mentioned before, C5a is a much more potent activator. Anaphylatoxins attract neutrophils, monocytes/macrophages, mast cells, and eosinophils toward the infection site and affect their adhesion. They act on mast cells, eosinophils, and basophils, causing their degranulation and release of histamine and other inflammatory molecules such as prostaglandins and leukotrienes. They also stimulate smooth muscles and endothelial cells to trigger vasodilation, increased permeability of small blood vessels, and muscle constriction [87]. C5a has a significant effect on neutrophils. It causes their migration, adhesion, production of ROS and neutrophil extracellular traps (NETs), and degranulation. Moreover, C5a inhibits the apoptosis process of neutrophils and

prolongs their functionality. C5a mediates the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα [88]. Due to the high potency of C5a, overproduction of C5a leads to severe problems. Sepsis is one of the most dangerous complications of the infection, with a mortality of even 50%. An overstimulated immune system produces massive amounts of pro-inflammatory molecules, including C5a. An imbalance of pro- and anti-inflammatory molecules leads to multiorgan failure and, in consequence, death of patients. C5a involvement in sepsis was shown using animal models and C5a agonists. Overproduction of C5a also might cause asthma and allergies due to the expression of TNFα and histamine by mast cells and basophils [89].

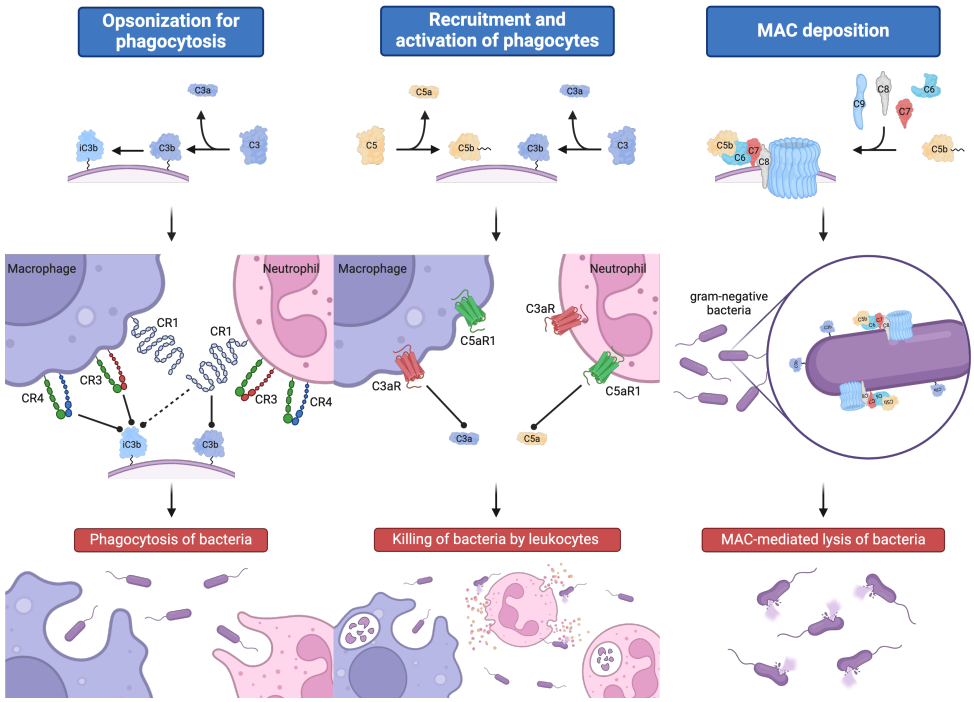

Figure 2. Primary outcomes of the complement system activation on the surface of gram-negative bacteria.

MAC deposition

The last step of the complement cascade activation is the formation of MAC. C5b binds C6, and next, both bind C7, which attaches the complex to the surface. Then, C8 joins the complex and inserts into the membrane. Finally, many copies of C9 create the pore

in the membrane, forming a complete complex. C6, C7, C8, and C9 proteins contain the MAC/perforin/cholesterol-dependent cytolysin (MACPF/CDC) domain. This domain is associated with the ability to form pores and is present in many other proteins [90]. Proteins containing this domain form pores by attaching to the membrane, forming the pre-pore, and finally changing the conformation of the first and second transmembrane regions (TMH1 and TMH2). As a result, the large β-barrel pore that pierces through the membrane is formed [91]. Intriguingly, C9 is the only component that may self-associate. To prevent unwanted C9 polymerization in the fluid phase, the TMH1 domain acts as an inhibitor. In the presence of C5b8, TMH1 unfurls and inserts into the membrane. This conformational change is accompanied by changes in TMH2 and movement in the helix-turn-helix structure. This mechanism makes the recruitment and sequential insertion of more C9 proteins possible [91].

Interestingly, physiological MAC is not a symmetrical, closed ring. Experiments with the polymerization of C9 proteins showed a symmetrical pore made of 22 monomers. However, cryogenic electron microscopy reconstruction of MAC showed that a typical MAC resembles more of a split-washer structure [92]. The structure can be closed or open, meaning there might be a 30 Å gap between the last inserted C9 and C6 [93]. This shows that MAC is a flexible structure that might change its confirmation. The MAC is a β-barrel structure that measures 305 Å in length. The outer diameter is 240 Å, while the inner diameter is 110 Å [92].

Dimensions of MAC are crucial for its function in bacteria killing. Complement activation and MAC deposition will occur on both gram-positive and gram-negative bacteria. However, the outcome of the deposition will be completely different. Gram-positive bacteria naturally resist MAC-mediated lysis due to their thick peptidoglycan layer protecting the inner membrane. The transmembrane domain of MAC has a length of 5 nm, while gram-positive bacteria peptidoglycan thickness can be 30-100 nm [94]. Therefore, MAC will be entrapped in the peptidoglycan layer and unable to deposit into the inner membrane to disrupt the cell [95]. Interestingly, MAC was observed to deposit in the specific regions of cells, compared to the ubiquitous deposition of C3 fragments. Nevertheless, there is still an ongoing debate about whether there is another function of MAC on gram-positive bacteria [96].

The deposition of MAC is more critical for the clearance of gram-negative bacteria. Individuals with protein deficiencies from the TP have a higher chance of infection caused by bacteria from the genus *Neisseria* [97]. That phenomenon is also observed during the treatment with complement inhibitors such as Eculizumab, which blocks

the TP. The gram-negative bacteria cell wall comprises a thin peptidoglycan layer surrounded by the inner and outer membranes [94]. The periplasmic space containing peptidoglycan is around 20 nm thick, while both membranes are only 5 nm thick each. This allows MAC to deposit tightly into the outer membrane and pierce through it with the transmembrane domain. The process of MAC deposition requires the presence of C5 convertase and then the secure association of C5b and C6 on the bacterial surface. Next, C5b-6 must attach quickly to C7 to continue with MAC formation successfully. If C7 is absent, C5b-6 can easily detach and fail to provide a foundation for the MAC [98]. The composition of the cell wall also influences the deposition process. Gram-negative bacteria produce LPS and outer membrane proteins that create uneven surfaces for MAC. Thus, the flexibility in the structure of MAC might be crucial [93]. Noteworthy is also the aspect of time necessary to form a pore. Rapid atomic force microscopy revealed that incorporating the first C9 into the C5b-8 complex is delayed by around 900 seconds. After that, the polymerization occurs fast, in around 112 seconds, resulting in just over six seconds per C9 molecule [99]. It is still unknown if the specific localization on the surface of C5 convertase and MAC matters in killing gram-negative bacteria. It is acknowledged that the deposition of MAC into the outer membrane creates a pore with a diameter of 10 nm. This damages the outer membrane, yet the question of how the inner membrane is damaged is still not fully answered. There was a suggestion that complement molecules or complete complexes such as C5b-6 could pass through the pore and form another MAC inside. The 10 nm pore is too narrow for complement proteins but big enough to pass smaller molecules such as antimicrobials. Lysozyme could potentially pass through the pore and disrupt peptidoglycan in the periplasm. It was also shown that antimicrobial peptides, such as nisin A, can penetrate the outer membrane using MAC pores [100]. Nisin A is a 3.4 kDa peptide that damages peptidoglycan in gram-positive bacteria [101]. The synergy with MAC allowed nisin to pass through the membrane and kill gram-negative bacteria. Another mechanism of how MAC kills bacteria might be simply the disturbance of osmotic balance within the cell. Distortion in the periplasmic protein concentration and inner membrane composition may result in extreme cellular stress that the repair mechanisms cannot diminish, leading to lysis [102].

The influence of MAC deposition on mammalian cells is intriguing. Complement deposition affects all neighbor surfaces, including host cells. Cells are usually protected from MAC by fluid-phase inhibitors Vn and Clu and membrane-attached inhibitor CD59. Some portion of MAC can still be inserted into host cells. Therefore, cells

subsequently use other mechanisms to remove MAC. They shed membranes with complexes or internalize them via endocytosis. Thus, the presence of MAC on mammalian cells is a temporary occurrence [61]. However, soluble MAC (sMAC) may induce or inhibit cell proliferation and apoptosis. In neutrophils and macrophages, it can release pro-inflammatory cytokines and degranulation. MAC pores cause the influx of Ca^{2+} ions into the cell, triggering downstream pathways. Interestingly, MAC is speculated to trigger the release of Ca^{2+} from the endoplasmic reticulum by forming a complex with GPI-anchored CD59 and interacting with G-protein coupled receptors [103]. Additionally, sMAC was shown to activate NLRP3 inflammasome, causing the production of IL-1β and IL-18 [104]. It was demonstrated that sMAC is present in body fluids under normal conditions, and its levels may vary due to the infection or autoimmune disease. Bacteria were shown to release MAC from their surfaces during infection [105]. It was also noted that levels of sMAC correlated with Eculizumab doses used to treat complement-related diseases. Therefore, sMAC was proposed to be used as a biomarker to monitor the progression of the disease [106].

Complement system crosstalk during infection

The complement system is not secluded from the general immune system. It cross-talks with different systems involved in pathogen detection and eradication. Complement cooperates with the adaptive immune system (B cells and T cells) and coagulation system and is involved in the signaling of Toll-like receptors.

Adaptive immune system

Complement modulates many functions of B cells, including their activation and differentiation, antigen presentation, and immunoglobulin class switching [107]. B cells express on their surface complement receptor 2 (CR2, CD21). CR2 is a 145 kDa glycoprotein, which resembles CR1. However, CR2 lacks domains binding C3b and C4b, therefore CR2 binds iC3b, C3dg, and C3d. It also recognizes the envelope protein of the Epstein-Barr virus, CD23, IFNα, and DNA [73, 81]. CR2 interacts with C3 fragments and forms a complex with CD19 and CD81. CR2:C3d complex induces B cell receptor signaling and activation of B cells. Thanks to the C3d opsonization, the activation threshold is lowered several-fold [80]. The interaction of CR2 is also

important in the generation of memory B cells. Interestingly, B cells express CR1 as well to bind C3b and C4b, but CR1, opposite to CR2, suppresses B cell activation. CR2 interaction with C3 fragments plays an important role in antigen presentation. B cells internalize C3-coated antigen, process it, and then present peptides on the MHC-II class receptors for T cells. Any blockade of CR2, e.g., with antibodies, reduces the uptake and presentation of the antigen. Moreover, CR2, with CD46 and C4BP, influences the immunoglobulin class switch. CR2 stimulates the production and secretion of IgE. C4BP acts similarly, binding CD40 and boosting IgE production. On the contrary, CD46 inhibits IgE production, modulating nuclear factor-κB (NFκB) activation [107].

Complement activation fragments also stimulate T cells. The C3 fragments opsonization is recognized by antigen-presenting cells, which present the antigen on their MHC receptors, which can activate T cells [107]. When binding to their specific receptors, C3a and C5a anaphylatoxins also stimulate T cells. This stimulation has a pro-inflammatory effect on T cells, causing cytokine production. Interestingly, no stimulation of C3aR and C5aR during T-cell activation leads to the induction of Treg cells. CD46 is an essential factor in T-cell activation. Interaction between Jagged1 and Notch1 controls T cell proliferation. CD46 inhibits the activation of T cells interacting with the Jagged1 protein [80]. Other complement receptors also influence T cell homeostasis. CD55 controls Th1 response and secretion of IFNγ [108]. CD59 has an immunomodulatory role, while CR1 reduces T cell proliferation and secretion of IL-2 [109, 110].

Coagulation system

Like a complement, the coagulation system is a cascade of serine proteases whose activation leads to clot formation. Clots of fibrin and platelets seal the damaged blood vessel, reducing blood loss and preventing bacterial infection. The coagulation cascade can be activated via two pathways: extrinsic and intrinsic. The extrinsic pathway starts with the exposition of tissue factor to blood when the blood vessel is damaged. Tissue factor forms a complex with Factor VII, and then both cleave Factor X. In the intrinsic pathway, Factor X is cleaved by the sequential activation of Factors XII, XI, IX, and with co-factor VIII. After the cleavage by any pathway, Factor X cleaves prothrombin into thrombin, which cleaves fibrinogen into fibrin. Fibrin forms a fiber network that forms a clot with platelets [111]. Coagulation can be regulated by protein C, which

inhibits thrombin formation, and by plasmin, which can degrade excessive fibrin build-up. Interestingly, Factor XII activates upon binding to a negatively charged surface, e.g., a bacterial surface containing LPS. Activated Factor XII stimulates kallikrein, which induces the release of the pro-inflammatory peptide bradykinin [112].

Complement and coagulation crosstalk in several places. In C3-deficient mice, researchers observed C5a levels of wild-type mice. They identified that thrombin acted as a C5 convertase to C5a. Interestingly, thrombin, plasmin, and factors Xa and Xia can cleave complement proteins, producing C3a and C5a, which influence neutrophil recruitment and inflammation [113]. Thrombin was also observed to produce non-canonical C5b since it cleaved C5 at the alternative site. This atypical C5b formed MAC, which remarkably was more potent than canonical complex [114]. On the other hand, plasmin can degrade C5b to limit MAC formation. Kallikrein showed the ability to cleave C3 and FB to stimulate AP activation. Platelets store complement proteins inside their granules, which, upon activation and degranulation, can be released into the environment. The complement system also regulates coagulation [115]. C1-INH effectively blocks kallikrein, thrombin, and Factors Xia and XIIa. LP proteases MASP-1 and MASP-2 cleave coagulation components and support clot formation. C4BP interacting with protein S regulates anticoagulation mechanisms since protein S loses the co-factor activity in Factors Va and VIIIa degradation [116].

The close relationship between complement and coagulation during the infection is evident. Coagulation entraps bacteria within clots, preventing their spread with blood. Simultaneously, complement opsonizes bacteria with C3 fragments and MAC and releases anaphylatoxins C3a and C5a. Captured bacteria are marked for phagocytosis and MAC-mediated lysis, while anaphylatoxins stimulate leukocytes for their pro-inflammatory functions. However, complement and coagulation amplification during sepsis may lead to pathological conditions. The depletion of complement and platelets characterizes sepsis. Complement overstimulation produces vast amounts of highly pro-inflammatory C5a. Inhibition of C5aR1 rescued mice in the meningococcal sepsis model. Moreover, C5 inhibition increased the survival of baboons during the sepsis model with *E. coli* from 0% to 80%. The inhibition reduced C3b and MAC deposition, cytokine production, coagulation consumption, and multiple organ damage in animals [115, 117].

Toll-like receptors

Toll-like receptors (TLRs) are type I transmembrane receptors acting as pattern-recognition receptors. They detect PAMPs, specific molecules present in pathogens (bacteria, viruses, fungi, and parasites). TLRs have been identified based on homologies with Toll receptors in *Drosophila*. Toll-deficient *Drosophila* was susceptible to fungi infection and unable to express anti-fungal protein [118].

There are ten receptors described in humans. They comprise extracellular leucine-rich domains responsible for PAMP binding, transmembrane domains, and intracellular Toll/interleukin-1 receptor (TIR) domains responsible for triggering signaling cascade. Each TLR has a different triggering ligand. TLR1, TLR2, and TLR6 bind lipoproteins, lipoteichoic acids, and peptidoglycan. TLR5 links to bacterial flagellin, while TLR4 binds to LPS. TLR3 recognizes double-stranded RNA, whereas TLR7 and TLR8 bind single-stranded RNA, and TLR9 recognizes DNA [118, 119]. The binding arrangement suggests that gram-negative bacteria will be mainly recognized by TLR4, while gram-positive bacteria will be recognized by TLR2 and dimers of TLR2/1 and TLR2/6. Noteworthy, the TLR2/1 recognizes lipopeptides present in gram-negative bacteria, while the TLR2-TLR6 recognizes lipopeptides in gram-positive bacteria. TLR5 signaling will depend on the bacterium having a flagellum. Intracellularly, nucleic acids from negative and positive bacteria will trigger intracellular TLRs.

Some TLRs are expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, and TL6), while some are associated with intracellular vesicles (TLR3, TLR7, TLR8, and TLR9). The vesicles are endoplasmic reticulum, endosomes, lysosomes, and endolysosomes. Interestingly, TLR11 was shown to be present extra- and intracellularly. Activation of TLRs by their specific ligand leads to the stimulation of NFκB/AP-1 or IRF3 to produce pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, TNFα, IFNα, and IFNβ [118, 119].

Complement and TLRs recognize similar PAMPs, such as LPS, zymosan, and bacterial DNA, and consequently trigger their respective cascades. The crosstalk results in increased pro-inflammatory cytokine expression, mainly due to the involvement of C3aR and C5aR1 in TLR signaling. Experiments on LPS-treated CD55-deficient mice demonstrated higher cytokine production in KO than wild-type mice due to the synergistic co-activation of complement and TLRs [120]. C3aR and C5aR1 support TLR signaling, activation of MAPK kinase, and downstream recruitment of NFκB and AP-1 to produce cytokines IL-1β, IL-6, or TNFα. Simultaneously, C5aR1 affects

kinases ERK and PI3K, which inhibit transcription factors IRF-1 and IRF-8, leading to the suppressed production of IL-12, IL-23, and IL-27. C5aR1 also reduces the expression of IL-17A. Interestingly, the blockage of pro-inflammatory cytokines was partially mediated by anti-inflammatory IL-10, whose expression complement has enhanced [120]. Furthermore, CR3 regulates TLR functions by stimulating ligand Mal, which activates MyD88 and MyD88-dependent cytokine production [121]. In contrast, CR3 causes MyD88 degradation, limiting cytokine secretion in macrophages [122].

TLRs have been shown to regulate complement expression. LPS-induced TLR4 induces the production of FB in macrophages. FB production was also suggested in colonic epithelial cells after the stimulation with double-stranded RNA analog and activation of TLR3 [123]. In macrophages, C3 production was possible by activating TLR4 and the liver X receptor with modified low-density lipoprotein [124]. TLRs also induce C3aR and C5aR expression by secreting cytokines such as IL-6 [125]. In general, TLRs influence complement expression, and in return, complement amplifies or inhibits TLR signaling.

An excellent example of complement-TLRs crosstalk is the evasion mechanism of *Porphyromonas gingivalis*. This gram-negative bacterium is responsible for periodontitis, leading to periodontal inflammation and bone loss [126]. *Porphyromonas* activates TLR2/1 and C5aR1 by producing locally C5a since the pathogen requires local inflammation for growth. The bacterium produces proteases – gingipains (HRgpA, RgpB, and Kgp) – which act similarly to C5 convertase and cleave C5 protein [126]. In neutrophils, TLR2/1-C5aR1 crosstalk leads to the ubiquitination and, consequently, degradation of MyD88, inhibiting pro-inflammatory downstream signaling. Instead of MyD88, the alternative trigger Mal is activated. Mal downstream activates PI3K, which leads to the inflammatory response. Interestingly, Mal-PI3K signaling triggered by *Porphyromonas* inhibits actin polymerization and phagocytosis of the bacterium [127]. Moreover, the inhibition of TLR2/1, C5aR1, or PI3K results in decreased growth and increased pathogen killing. In macrophages, TLR2/1-C5aR1 crosstalk results in the overproduction of cAMP, which activates kinase PKA and further inhibits synthase GSK3β. As a result, the production of antimicrobial nitric oxide is blocked. Moreover, the crosstalk also regulates cytokine expression in macrophages. It downregulates the secretion of IL-12 but upregulates the expression of IL-1β, IL-6, and TNF [127, 128]. Additionally, *Porphyromonas* utilizes recognition by CR3 and its crosstalk with TLRs, leading to the cytokine production and survival of bacterium inside macrophages. In

contrast, in dendritic cells, neither C5aR1 nor CR3 helps the pathogen to survive. Strikingly, C5aR1 and C3aR in dendritic cells enhance the killing of the bacterium [128].

Intracellular complement system

The canonical view of complement has been that the system functions exclusively extracellularly and systemically in serum. With the increasing number of studies showing local expression of complement proteins by immune and non-immune cells, more evidence for non-canonical functions of complement has been presented [129]. This led to the discussion about the presence of intracellular complement and, finally, to studies describing complement functions inside the cell [130]. To date, several complement proteins such as C3, C5, C1q, and FH, as well as receptors for C3a and C5a, have been described to function intracellularly [131].

C3 protein is, by far, the most-studied intracellular complement molecule. C3 is associated with the pathogen-sensing function; thus, a similar function was discovered intracellularly. C3-opsonized adenovirus particles were absorbed by non-immune cells, where C3 triggered NFκB and the release of cytokines [132]. Similarly, internalized bacteria covered with C3 fragments were targeted by xenophagy, a type of autophagy directed towards bacteria. Invasive pathogen *Listeria monocytogenes* was cleared via autophagy, while *Shigella flexneri* and *Salmonella* Typhimurium survived inside the cell due to the secretion of proteases IscP and PgtE that cleaved deposited C3 [133]. C3 fragments on bacteria interacted with the ATG16L1 protein involved in the autophagy activation. ATG16L1 interacts with ATG12 and ATG5 and forms a complex. ATG complex causes ubiquitin-like protein LC3-I to LC3-II lipidation, which triggers autophagosome formation [134]. Despite the exact mechanisms being unknown, it indicated that C3/C3b/iC3b could be transported inside the cell and trigger a pro-inflammatory reaction and bacterial killing.

Remarkably, C3 can be expressed intracellularly in CRISPR/Cas9-modified A549 cells. Knockout of the first ATG site results in the C3 present in the cytosol. Notably, the modified cytosolic C3 from ΔATG1 A549 cells can detect bacteria, e.g., *Staphylococcus aureus*. Bacteria were opsonized with a non-canonical 65-kDa C3 fragment, which was recognized by professional phagocytes once the bacteria were recovered from A549 cells

[135]. This shows the importance of intracellular C3 in pathogen detection. However, factors cleaving C3 inside the cell are undetermined.

Other complement proteins are also involved in intracellular cell functions. C1q can interact with gC1qR present within mitochondria. Thus, C1q may regulate cell metabolism by interacting with mitochondria, e.g., in $CD8^+$ T-cells. FH was shown to co-localize with C3 in endosome/lysosome. Therefore, FH can intracellularly mediate C3 cleavage by lysosomal enzymes, e.g., cathepsin L. FH expression was also correlated with worse outcomes in renal cell carcinoma or lung adenocarcinoma [131]. Typical CD59 has a GPI anchor that attaches it to the membrane. However, CD59 isoforms present in cells do not contain GPI. CD59-IRIS isoforms are retrotranslocated into the cytosol from the endoplasmic reticulum. In pancreatic β-cells, CD59 isoforms mediate insulin secretion [136].

While the existence of intracellular complement proteins is now accepted, the concept of "complosome" i.e. the presence of complement proteins intracellularly engaging together in a canonical manner and cellular localization of these processes, is still debatable. The acidic pH within endosomes and the reducing nature of the cytosol raise the question of whether complement proteins will retain their properties. Lowered pH may interfere with the convertase function, and enzymes, such as cathepsins, may be involved in the intracellular cleavage of complement proteins. Moreover, canonically expressed C3 has disulfide bonds that are extremely important for its structure and function [137]. Enzymes such as reduced glutathione may damage disulfide bonds within the C3 proteins, rendering them inactive. Therefore, intracellularly expressed proteins may either not have bonds formed during expression or have them reduced after release from the endoplasmic reticulum. The reduction of the bond may also mediate non-canonical functions within the cell. However, the bond within the C3 protein is only exposed during activation and conformational changes so that it may remain protected from the environment most of the time. In addition, once C3 is attached to the surface of a pathogen, it cannot be degraded by reducing agents [131].

As for the source of intracellular complement, there are several possibilities based on the proposed source of C3. First, proteins may be transported back into the cytosol, as C3 is transported on the surface of a pathogen. Alternatively, they could be actively internalized by cells. Second, proteins could be secreted within the cell without a signal peptide, preventing their transport outside. Eventually, proteins may interact with proteins involved in the secretion process within the endoplasmic reticulum or Golgi

apparatus. Consequently, proteins are secreted directly into the cytosol without post-translational modifications such as glycosylation [131].

Human pathogens

The human body is constantly exposed to pathogens. One study estimated the number of human pathogens for 1513 species identified before 2021. Out of the total number, 73% were considered established pathogens as they caused at least several reported cases; 27% that caused less than three reported infections have been regarded as putative pathogens [138]. Interestingly, out of 24 classes, Gammaproteobacteria and Actinomycetia contained the most pathogenic species. The Gammaproteobacteria class includes the most popular pathogens, such as *E. coli*, *K. pneumoniae*, *Salmonella*, *P. aeruginosa*, and *A. baumannii*. The Actinomycetia class includes *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Corynebacterium diphtheriae*.

Antimicrobial resistance and mortality

The scientific community studied these pathogens, trying to develop methods to treat bacterial infections. With the discovery of penicillin in 1928 by Alexander Fleming, we have entered the era of antibiotics [139]. Despite the enormous progress in antibiotic production, barely 100 years after penicillin's discovery, we must face the severe issue of antimicrobial resistance (AMR) [140]. Primarily, resistant pathogens have been classified as multidrug-resistant (MDR) as they acquired resistance to at least one agent in three or more antimicrobial categories. However, the escalation of resistance created additional classifications such as extensively drug-resistant (XDR), meaning resistant to at least one agent in all but two or fewer antimicrobial categories, and pandrug-resistant (PDR), meaning resistant to all agents in all antimicrobial categories [141].

In 2008, Louis Rice suggested classifying some of the most dangerous pathogens, based on their virulence and AMR, as ESKAPE bugs [142]. He created the acronym using the names of pathogens:

***E**nterococcus faecium*
***S**taphylococcus aureus*
***K**lebsiella pneumoniae*
***A**cinetobacter baumannii*
***P**seudomonas aeruginosa*
***E**nterobacter* spp.

In 2017, the WHO reported the problem with AMR and the need to develop novel treatment options against resistant pathogens [143]. Priority 1: critical status was given to carbapenem-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa*, and carbapenem-resistant, third-generation cephalosporin-resistant Enterobacteriaceae. WHO published an updated list in May 2024 [144]. The meta-analysis of AMR estimated that in 2019, approximately 4.95 million deaths could be associated with AMR, including 1.27 million deaths that could be attributed to AMR. The leading AMR pathogens, *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, were responsible for 3.57 million deaths associated with AMR, with 929,000 deaths attributed to AMR [140]. Another meta-study focused on the general mortality associated with 33 pathogens independently of their AMR. They estimated that in 2019, approximately 13.7 million infections led to deaths globally, alongside 7.7 million deaths related to studied pathogens. These 33 bacteria were correlated with 13.6% of all deaths worldwide and 56.2% of all infection-related deaths in 2019. The deadliest pathogens were *S. aureus*, *E. coli*, *S. pneumoniae*, *K. pneumoniae*, and *P. aeruginosa* [145]. In both studies, lower respiratory tract infections and bloodstream infections were the leading causes of death [140, 145].

Complement system evasion mechanisms

The complement system is a very potent mechanism against microorganisms. Many human pathogens, including bacteria, viruses, fungi, and parasites, are known to develop evasion strategies from the complement system. Complement activation results in several outcomes, and the complement cross-talks with several other systems. Therefore, bacteria such as *Staphylococcus aureus* or *Borrelia burgdorferi* utilize several mechanisms to evade complement activation.

Targeting the activation

Staphylococcus aureus expresses cell-wall Protein A that binds human IgG. Protein A binds the Fc domain of antibodies, which results in the coating of the bacterium with IgG but in the incorrect configuration. That prevents the C1 complex from activating the CP and phagocytic cells from activating Fc receptors. *S. aureus* can also bind C1q using Cna protein, which blocks the interaction of C1q with C1r [146]. Similarly, *Borrelia burgdorferi* inactivates the C1 complex by non-covalently binding C1r using lipoprotein BBK32. Moreover, *Bordetella pertussis* interacts with C1-INH using Vag8 protein, which leads to the degradation of C1r, C1s, and MASP-2 and depletion of complement. *Neisseria meningitis* and *Escherichia coli* K1 produce capsules poorly recognized by human antibodies, thus escaping from the complement activation [147]. Interestingly, *Moraxella catarrhalis* has been shown to bind the cartilage oligomeric matrix protein (COMP) using the UspA2 protein. COMP may act as a CP inhibitor interacting with C1q [77].

Targeting the convertases

S. aureus successfully blocks C3 convertases, reducing opsonization with C3 fragments. Secreted Eap protein binds C4b, preventing further interaction with C2 and C2b, inactivating CP and LP. Furthermore, *S. aureus* produces SCIN protein that hinders all C3 convertases in a non-active state. SCIN was shown to bind C3b and Bb to make them non-functional [146]. Additionally, proteins Efb and its homolog Ecb inactivate C3 convertase by preventing FB cleavage by FD. Efb and Ecb were also proven to block C5 convertase by interacting with C3d. C5 convertase is also blocked by *S. aureus* using SSL-7 secreted protein that binds C5, preventing its interaction with convertase [146]. Moreover, ClfA, a cell wall protein, was recognized as an FI-binding protein, which reduced C3 convertase activation. *M. catarrhalis* can bind serum C3 using surface proteins UspA1 and UspA2, as can a periodontal pathogen *Filifactor alocis* using FACIN protein [148, 149].

Targeting the inhibitors

The most common pathogen evasion strategy is to bind the fluid phase inhibitors FH and C4BP [147]. Bacteria acquire FH by primarily binding CCPs 6-7 and 19-20, which are responsible for the binding to host cells. Thus, CCPs 1-4 can interact with C3b to dampen the activation. *S. pneumoniae* uses two surface proteins, Hic and PspC, to bind FH, while *B. burgdorferi* expresses many factors, such as CspA, CspZ, ErpA,

ErpP, and OspE, that bind CCP 20 of FH. The mechanism of FH acquisition in *N. meningitis* and *N. gonorrhoeae* is interesting, as they produce sialic acid on their LOS and surface proteins to mimic the host surfaces [150]. Similarly, pathogens bind to α-chains of C4BP. *M. catarrhalis* acquires C4BP using surface-expressed UspA1 and UspA2 proteins. *N. meningitis* and *N. gonorrhoeae* bind the inhibitor using PorA and Por1A proteins, respectively. *S. pneumoniae* uses LytA, PspC4.4, and the surface exposed enolase to bind C4BP [147, 151].

Targeting the MAC formation

Pathogens also develop strategies to avoid MAC deposition. *B. burgdorferi* produces a CD59-like protein that inhibits MAC formation. It also produces a surface protein, CspA, which interacts with C7 and C9, reducing C9 polymerization and MAC formation [152]. *H. influenzae*, *P. aeruginosa*, and *M. catarrhalis* inhibit MAC deposition by acquiring soluble inhibitor Vn [149, 151].

Targeting the proteins

Pathogens can also degrade complement proteins to prevent the activation. Especially good at cleaving are periodontal bacteria. *Porphyromonas gingivalis* secretes gingipains HRgpA, RgpB, and Kgp, which cleave C3, C5, and C5. *Tannerella forsythia* produces protease mirolysin, which degrades MBL, ficolins, C4, and C5 [153]. Furthermore, *S. aureus* expresses protease aureolysin that cleaves C3. Also, pathogens *Shigella flexneri* and *Salmonella* Typhimurium secrete proteases IscP and PgtE that degrade deposited C3, allowing pathogens to survive intracellularly [133].

Acinetobacter baumannii

General information

In 1911, Dutch microbiologist Martinus Beijerinck isolated bacteria from soil. He grew bacteria in a minimal medium enriched with calcium acetate; thus, he called them *Micrococcus calcoaceticus* [154]. Over the next few decades, bacteria were isolated several times and called, with many different names, such as *Diplococcus mucosus*, *Micrococcus calcoaceticus*, *Alcaligenes haemolysans*, *Bacterium ani*tratum, *Mima polymorpha*, *Herellea vaginicola*, *Moraxella lwoffi*, *Moraxella lwoffi* var. *glucidolytica*, *Achromobacter anitratus*, *Achromobacter mucosus*, and *Neisseria winogradskyi* [155]. In 1954, the name *Acinetobacter* was proposed, meaning non-motile (*ακινετοσ* in Greek), since the bacterium lacked flagellum, but only by 1971 the name was officially recognized. The genus *Acinetobacter* was ultimately organized in 1986 thanks to Philippe Bouvet and Patrick Grimont and their work on DNA-DNA hybridization [156]. Currently, over 70 distinct species have been identified. Interestingly, *Acinetobacter* species are very closely related and difficult to distinguish phenotypically. Thus, some species are often grouped into the *A. baumannii-A. calcoaceticus* complex. Noteworthy, *A. tjernbergiae* has been named to honor Ingela Tjernberg, a microbiologist who used to work at Lund University and contributed to the taxonomy of *Acinetobacter* [157].

Genus *Acinetobacter* is classified in the class Gammaproteobacteria, family *Moraxellaceae*. They are gram-negative, strictly aerobic bacteria, typically of coccobacillus shape. Despite being considered non-motile, they display a twitching motility. *A. baumannii* is the most clinically relevant species causing opportunistic infections. The source of *A. baumannii* is still not well understood. The bacterium has been isolated from the natural environment (water, soil, food, sewage, human and animal skin), yet it is mainly associated with the hospital environment [155].

Clinical importance

A. baumannii is well known for its extreme virulence and resistance to antibiotics. In 2008, it was included in the ESKAPE list [142], while in 2017, WHO classified the carbapenem-resistant bacterium as the top priority for developing new antibiotics [143]. In the updated list version, *A. baumannii* is still classified in the critical group but is in third place behind *Klebsiella pneumoniae* and *E. coli* [144]. Remarkably, until

the 1970s, *A. baumannii* was successfully treated with common antibiotics, but the AMR has been spreading quickly. The pathogen gained notoriety in the early 2000s after causing an outbreak in a US military hospital in the Middle East and earning the nickname Iraqibacter [158]. During the Ukraine conflict, an increase in infections caused by MDR *A. baumannii* has been observed [159].

A. baumannii is an opportunistic human pathogen, thus mostly dangerous for immunocompromised people such as intensive-care unit (ICU) patients. The bacterium accounts for around 2% of infections in the US and Europe, almost 5% in Asia, and around 20% of ICU infections worldwide. It causes ventilator-associated pneumonia, bloodstream infections, meningitis, and wound infections (**Figure 3**). The average mortality is estimated at around 50%, but a meningitis infection can be fatal even in 70%, while pneumonia even in 80% of cases [160]. *A. baumannii* can easily colonize skin, airway ducts, lungs, and abiotic surfaces such as beds, sinks, tables, and medical equipment. Worryingly, it is known for its persistence and resistance to environmental/anthropogenic stressors, increasing its ability to colonize and cause infections [161].

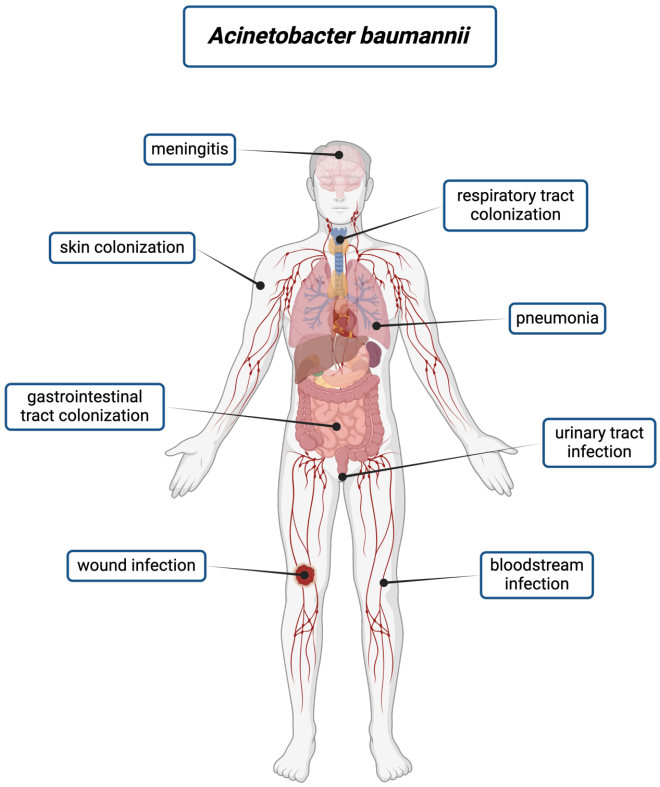

Figure 3. *A. baumannii*-mediated diseases and colonized surfaces.

Virulence factors

A. baumannii expresses many virulence factors involved in adhesion, serum resistance, cytotoxicity, bacterial competition, and survival (**Figure** 4).

General virulence

A. baumannii produces a polysaccharidal layer that protects bacteria from environmental stress, complement, phagocytosis, antimicrobials, and antibiotics. Genes involved in capsule production are organized in the K locus, and currently, over 100 capsule types have been identified in *A. baumannii*. Knocking out the capsule decreases the pathogen's survival [162].

As a gram-negative bacterium, *A. baumannii* expresses LPS or LOS. Typical LPS (smooth LPS) contains attached to the outer membrane lipid A, core oligosaccharide, and O antigen. *A. baumannii* lacks O antigen and thus expresses LOS (rough LPS) [163]. LOS triggers the activation of TLR4 and the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα. LPS/LOS is targeted by colistin. Interestingly, lack of LPS/LOS decreases membrane integrity but renders bacteria resistant to colistin. LPS/LOS is also a key complement target; thus, modifications can reduce activation [164].

Outer membrane proteins (OMPs) called porins are β-barrel proteins involved in adhesion and invasion, biofilm, virulence, and antibiotic resistance. OmpA is the most critical OMP that can trigger autophagy in host cells via MAPK/JNK signaling, induce mitochondrial degradation, and activate NLRP3, contributing to the intracellular survival of *A. baumannii*. Other identified OMPs such as CarO, Omp33, and OmpW contribute to the pathogen virulence similarly to OmpA [163, 164].

Outer membrane vesicles (OMVs) are secreted by gram-negative bacteria 20-200 nm in diameter, spherical vesicles containing virulence factors such as LPS/LOS, OMPs, periplasmic proteins, DNA, and RNA [165]. OMVs transfer virulence factors to host cells, triggering a pro-inflammatory response and causing cytotoxicity. The exact composition of factors is still unknown since they carry many proteins and peptides inside. In *A. baumannii*, OMVs are involved in the spread of AMR, transporting resistance plasmids between bacteria [166]. OMVs are also suggested to carry iron and iron-transporting proteins to support the virulence of other bacteria [167].

Although *A. baumannii* does not produce flagella and is not considered motile, it expresses Type IV pili, which allow for a crawling-like movement called twitching. A similar mechanism can be observed in other bacteria, e.g., *Pseudomonas aeruginosa.* Type IV pili, besides twitching motility, are involved in DNA uptake, virulence, and biofilm formation, aiding bacterium persistence in the clinical environment [168]. Another type of pili produced in *A. baumannii* is Csu pili, which are responsible for biofilm, surface adhesion, and binding to host cells such as A549 epithelial cells [164].

Complement evasion

Several reports indicate the potential involvement of virulence factors in complement evasion. CipA is an outer membrane protein binding plasminogen and degrading fibrinogen and C3b. CipA was also speculated to degrade C3b and C4b by interacting

with Factor I [169]. Tuf is a cytoplasmic elongation factor and chaperone protein. However, Tuf can be translocated to the surface and, similar to CipA, bind plasminogen [170]. Phospholipases C and D (PLC, PLD) degrade phospholipids and can damage host cell membranes. PLC1 and PLC2 contribute to cytotoxicity in erythrocytes and A549 cells and virulence against *Galleria mellonella* larvae [171]. The cooperation of PLD1, PLD2, and PLD3 is necessary for the invasion of A549 cells, and only a knockout of all of them reduced the pathogen's virulence. However, a knockout of one of the PLDs decreased serum survival [172]. SurA1 is a surface antigen involved in virulence and bacterial fitness. SurA1 mutant was shown to have reduced survival in human serum and the *G. mellonella* infection model [173]. OmpA was shown in one report to bind FH, contributing to the complement resistance [174].

Secretion systems

A. baumannii uses several secretion systems (T1SS, T2SS, T5SS, T6SS) to produce virulence factors [175]. T1SS is a ubiquitous system that transports adhesins, enzymes, iron-scavenger proteins, and toxins from cytosol outside the bacterial cell. T1SS secretes BAP and BAP-like BLP1 and BLP2 surface proteins. These proteins are directly involved in biofilm formation and adhesion to epithelial cells and abiotic surfaces. Due to the low isoelectric point (pI 2.9), BAP is also speculated to be involved in the AMR. T1SS is directly involved in the virulence against *G. mellonella* larvae. It also secretes RTX toxin, but its function is unknown [175]. T2SS transports proteins from the periplasm into the environment. Lipases A and H produced by T2SS are involved in the survival and colonization during the infection. CpaA is a metalloendopeptidase whose secretion is regulated by the chaperone CpaB. CpaA is said to cleave Factors V and XII, disrupting coagulation and complement systems. It is also speculated that it might degrade CD46 and CD55 due to the ability to cleave O-linked glycoproteins. Interestingly, CpaA is expressed by some clinical isolates but not laboratory strains like ATCC 19606 [176]. T5SS is located in the outer membrane and presents the active domain extracellularly. *A. baumannii* has only two types of T5SS, type Vb and Vc. Type Vc, Ata protein, is a crucial adhesin found in some isolates, binding to extracellular matrix (ECM) proteins such as collagen types I, III, IV, and V, and laminin, and to host glycans including galactose and N-acetylglucosamine. Ata is involved in the invasion and apoptosis of host cells [177]. Type Vb, a CDI complex made of CdiA and CdiB, is engaged in the competition between *Acinetobacter* species and in biofilm formation. The interaction with host cells so far has not been identified [178]. T6SS is a syringe-like complex injecting toxins into competing bacteria and host

cells. Competition with other bacteria is an essential step in establishing a successful infection. T6SS was important in *G. mellonella* infection and survival in the presence of serum [179].

Survival mechanisms

Iron and zinc are essential metals for bacterial metabolism, DNA replication and repair, and enzymatic reactions. They are also crucial for virulence as their concentration regulates protein expression [163]. However, bacteria have to balance the uptake of metals as they can be toxic in high concentrations. In the human host, most iron is localized within hemoglobin or bound to proteins such as ferritin, lactoferrin, and transferrin. *A. baumannii* has to lyse erythrocytes first to release hemoglobin, which PLC1 and PLC2 can do [163]. Hemoglobin is then bound by a secreted HphA protein, which transfers heme into the cell via the outer membrane receptor HphR. Intracellularly, heme oxygenase HemO cleaves heme to release free iron [180]. Interestingly, laboratory strain ATCC 17978 could not utilize the HemO system, implying that more virulent strains use this system [181]. To retrieve iron from iron-binding proteins, *A. baumannii* produces three types of siderophores, the metal chelators directly involved in pathogen survival and virulence. Acinetobactin, baumannoferrin, and fimsbactin transport iron into the cytosol via outer membrane receptors BauA, BfnH, and FbsN, respectively [182]. Each siderophore alone is sufficient to provide iron for cells. Acinetobactin is a highly conserved chelator with two pH-dependent isoforms that can acquire iron from transferrin and lactoferrin. Acinetobactin is essential for virulence as the mutant had decreased virulence in mouse and *Galleria* models. Acinetobactin mutant also had reduced intracellular survival and lower apoptosis rate in A549 cells compared to the wild-type ATCC 19606. Interestingly, acinetobactin is a versatile chelator that can bind many divalent cations, such as iron, zinc, manganese, copper, nickel, and cobalt [182, 183]. Baumannoferrin was first identified in *A. baumannii* AYE. This strain can survive with only baumannoferrin as it lacks acinetobactin, while ATCC 19606 produces all three siderophores yet still needs acinetobactin. Baumannoferrin has two isoforms, A and B, contributing to pathogen virulence [184]. Fimsbactins are rare as they are made by around 10% of *A. baumannii* strains, e.g., ATCC 17978. Fimsbactin A is thought to be the main siderophore, while forms B through F are regarded as synthesis intermediates. Fimsbactins' scarcity in *A. baumannii* strains is explained that they serve as a substitute in situations where acinetobactin is absent [185]. *A. baumannii* acquires zinc using the ZnuABC system. The complex is essential for mice pulmonary infection

and is regulated by the intracellular zinc concentration. The limitation of zinc contributed to the decreased AMR, which explains that zinc is necessary for the activity of enzymes, and carbapenemases are metalloenzymes that require zinc to cleave antibiotics [186].

Quorum sensing is an important system that stimulates the expression of the capsule, LPS, biofilm, and motility and adhesion factors. It is also said to be a critical factor in the AMR. The system in *A. baumannii* is homologous to the LuxI/LuxR system found in most gram-negative bacteria. It is based on the AbaI synthase that secretes AI-1 signaling molecules, derivatives of N-acyl homoserine lactones, outside the cell. AI-1 peptides bind to the receptor AbaR, and the complex stimulates gene expression and virulence [187]. BfmS/BfmR is a system that controls the pathogen response to environmental stress, such as desiccation or antibiotics. Upon the stress stimuli, BfmR is phosphorylated and causes gene upregulation. BfmS/BfmR induces the production of capsule, pili, and OMPs, therefore regulating biofilm formation, adherence to host cells, and complement resistance. Mutated bacteria showed decreased virulence against *G. mellonella* and decreased AMR to some antibiotics. The system was also shown to combat ROS production during the desiccation by producing catalases [188, 189]. *A. baumannii* uses RecA protein to neutralize the DNA damage. RecA takes part in homologous recombination and SOS mutagenesis. It forces the DNA exchange between ssDNA and dsDNA. It can also stimulate error-prone polymerases that replicate damaged DNA, rescuing cell survival. RecA mutants showed significantly worse macrophage survival and less virulence in the mouse infection model [190].

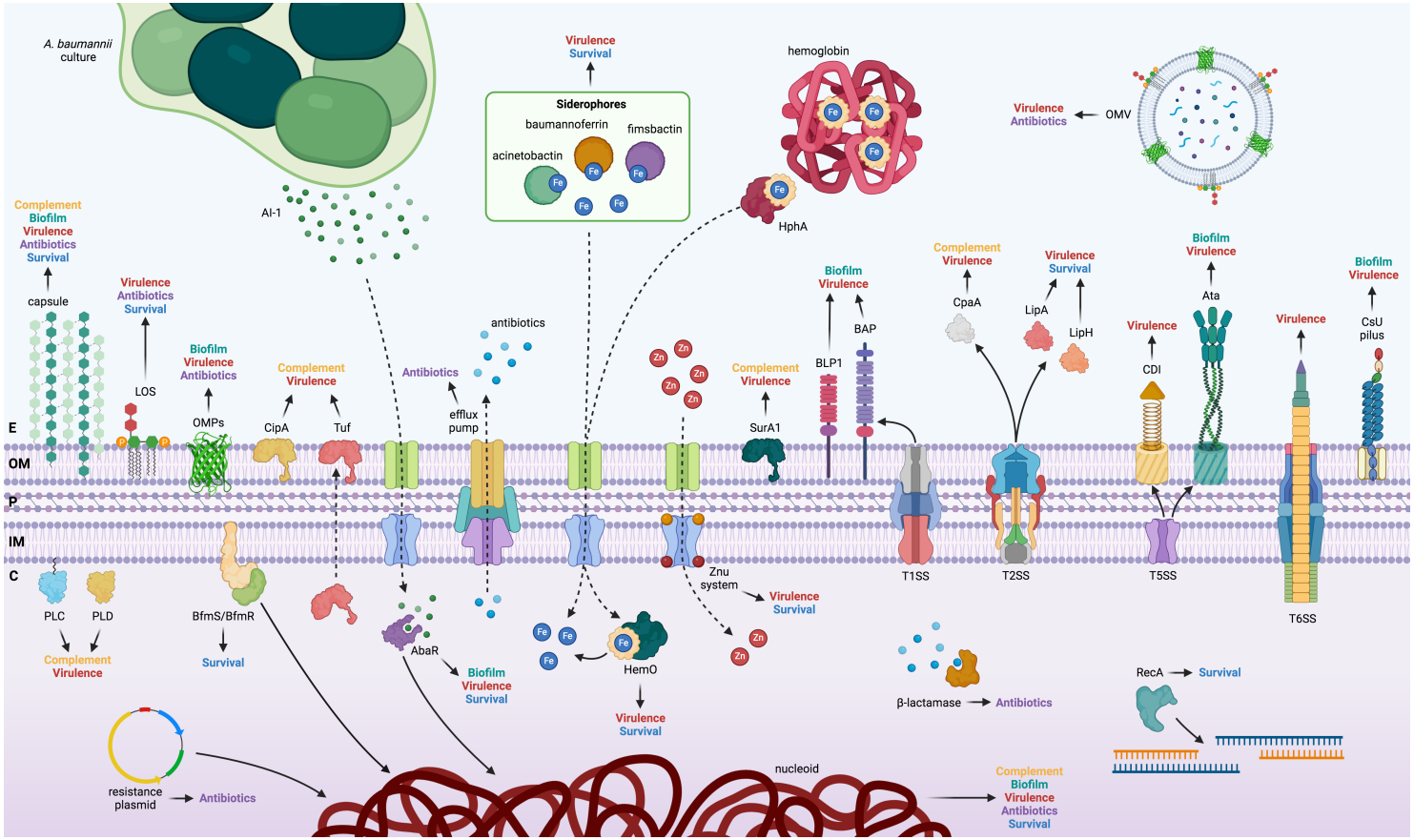

Figure 4. *A. baumannii* virulence factors involved in the complement resistance, biofilm formation, host virulence, antibiotic resistance, and survival (E – environment, OM – outer membrane, P – peptidoglycan, IM – inner membrane, C – cytoplasm).

Antibiotic resistance

A. baumannii is known for its AMR. The pathogen was shown to be resistant to β-lactams (penicillins, cephalosporins, carbapenems, monobactams, and β-lactamase inhibitors), aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol, and polymyxins. MDR *A. baumannii* is the primary pathogen causing infections. However, XDR and PDR isolates have been reported, indicating how serious the problem *Acinetobacter* is. Truly impressive is the genetic plasticity of the pathogen, as it needed roughly 50 years to become a worldwide threat [191].

β-lactamases

β-lactams are similar in structure to peptidoglycan precursor molecules. Penicillin-binding proteins mistakenly bind β-lactams, which inhibits the process of peptidoglycan formation, leading to cell wall damage and bacterial death.

There are four classes of β-lactamases: A, B, C, and D [192]. Class A provides resistance against penicillin, cephalosporins, monobactams, and carbapenems. *A. baumannii* expresses several Class A enzymes, including TEM, SHV, PER, CTX-M, and CARB. Class B, also called metallo-lactamases, require zinc to be active and can cleave almost all β-lactams. Class B includes IMP, VIM, SIM, and NDM, where NDM-1 is responsible for the extended AMR and PDR in *Acinetobacter*. Class C, called cephalosporinases, provides resistance against third-generation cephalosporins. Class D, called oxicillinases or OXA, is the main cause of the carbapenems resistance in *Acinetobacter* and cleaves all β-lactams. Enzymes include OXA-23, OXA-24, OXA-51, and OXA-58 families. OXA-23 was the first identified and is currently the most often occurring, whereas OXA-51 was found to express in *A. baumannii naturally* but with weak hydrolyzing activity [191, 193].

Outer membrane proteins

OMPs are non-specific porins that can aid the AMR. Dampened expression or lack of OMPs increases the AMR in *A. baumannii*. Knockout of OmpA, CarO, and Omp33 increased the carbapenems and chloramphenicol resistance.

Efflux pumps

A. baumannii uses an efflux system to pump out antibiotics from cells. The best-studied RND superfamily comprises AdeABC, AdeDE, AdeIJK, AdeFGH, and AdeXYZ pump

systems [193]. The expression of AdeABC pumps increases carbapenem and chloramphenicol resistance. AdeRS complex controls the AdeABC expression, and the mutation within the complex can significantly boost the pump expression. Thanks to the AdeABC pump, the pathogen can endure the effects of ethanol, benzalkonium chloride, and chlorhexidine. Overexpression of AdeDE helps to pump out ceftazidime and rifampicin. AdeIJK pumps can increase the resistance to carbapenems, cephalosporins, fluoroquinolones, tetracycline, neomycin, and tigecycline. Pumps are also said to play a role in regulating the lipid composition of the cell membrane. AdeFGH is found only in a subset of isolates, and it exports trimethoprim, chloramphenicol, fluoroquinolones, tetracycline, tigecycline, and clindamycin. AdeXYZ is a homologous system to AdeIJK [194]. *A. baumannii* also produces efflux pumps from different families. CraA and AmvA belong to the MFS superfamily and export chloramphenicol and erythromycin. AbeM from the MATE family and AbeS from the SMR family improve resistance against antibiotics, such as aminoglycosides, fluoroquinolones, and chloramphenicol [195].

Colistin resistance

Worryingly, *A. baumannii* is becoming resistant to polymyxins. Colistin is often used as a last-resort drug during problematic infections. Despite being nephrotoxic, polymyxins are used more often because of the AMR, which unfortunately raises the chance of developing resistance to them [191].

Polymyxins target bacterial LPS working in a detergent-like fashion. Positively charged antibiotics interact with negatively charged lipid A in LPS. Bacteria stop producing lipid A and lose LPS to counter the colistin effect. Interestingly, lacking LPS makes them antibiotic-resistant, but they can be killed by neutrophil-produced lysozyme and become more serum-sensitive [196]. The pathogen can also modify the LPS, mutating the *pmrC* gene, which removes the negative charge in lipid A and increases the colistin resistance. Unfortunately, resistance can also be passed via plasmids, as reports showed that the polymyxin-resistance *mcr* gene can be exchanged between bacteria [197].

Treatment options

On average, 80% of *A. baumannii* clinical isolates are carbapenems-resistant worldwide. In Europe, that number is relatively low, about 15%. However, it can reach even 100% of isolates in Southeast Asia. Therefore, the use of carbapenems as

monotherapy might work only in areas with a low percentage of resistance [155]. They can always be used with other antibiotics, such as β-lactamase inhibitors or polymyxins. Colistin is still effective against *Acinetobacter* infections. Nevertheless, resistance has been reported, with around 10% of isolates being resistant in the US and Europe and over 50% in India and Iran. Unfortunately, colistin's side effects have to be considered during the treatment. However, polymyxin B might also be considered as it is less toxic and potentially effective against infections [198].

One can mention phage therapy, Acinetobacter-specific antibodies, and prevention with vaccines as alternative options. The possibility of using bacteriophages is getting more popular due to the high AMR. *In vitro* studies showed the efficacy of phage against *A. baumannii*. Interestingly, the pathogen can develop a resistance against phages, which increases the antimicrobial sensitivity as it may lose a capsule, a target for phages and protection from antibiotics [199]. Monoclonal antibodies against *A. baumannii* are at a very early stage of development. The use of antibodies might potentially increase the opsonization and phagocytosis of bacteria. Additionally, antimicrobial peptides showed optimistic results against *A. baumannii*. However, they are still far from being used clinically due to their potential host cytotoxicity and production costs [200]. Finally, vaccines could potentially protect from *A. baumannii* infections. Live-attenuated bacteria, OMPs, OMVs, and polysaccharide capsules were tested as immunogens. Despite promising pre-clinical results, no proposed vaccine entered clinical trials. Nevertheless, with the development of mRNA-based vaccines, there is a potential for new studies [160].

Klebsiella pneumoniae

General information

Klebsiella pneumoniae was first isolated in 1882 by a German microbiologist, Carl Friedländer [201]. Bacteria were later named to honor another microbiologist, Edwin Klebs [202].

Genus *Klebsiella* is classified in the class Gammaproteobacteria, family *Enterobacteriaceae*. *Enterobacteriaceae* also includes other important human pathogens such as *E. coli*, *Salmonella*, and *Shigella*. *K. pneumoniae* is a gram-negative, encapsulated, nonmotile, and rod-shaped bacterium. It can be found in the soil, water, and many plants. It can also colonize and live as a commensal in animals. In humans, *Klebsiella* colonizes mucosal surfaces of the respiratory and gastrointestinal tracts. From these niches, the pathogen can metastatically spread in the organism and cause opportunistic infections [202].

Clinical importance

Similar to *A. baumannii*, *Klebsiella* is known for its virulence and AMR. *Klebsiella* is on the ESKAPE list [142] and is classified in the WHO report as a critical pathogen [143]. Recently, in the updated list from 2024, WHO classified *Klebsiella* at the top of the list [144]. The isolate of *K. pneumoniae* carrying the first KPC carbapenemase was identified in 1996 in North Carolina, US. Since then, several new carbapenemases have been identified [203]. During the ongoing conflict in Ukraine, we observed increased infections caused by *K. pneumoniae*. Worryingly, isolated pathogens displayed MDR, XDR, and even PDR being resistant to all available antimicrobials, including colistin [159].

K. pneumoniae has two different types, Classical (CKP) and Hypervirulent (HvKP), where the CKP type is characterized by higher resistance, and the HvKP type is less resistant but produces more virulence factors [203]. That suggests that HvKP infection might have worse initial symptoms, yet it could be treated with antibiotics due to the lower AMR. The CKP is responsible for typical hospital-acquired infections such as pneumonia, urinary tract infections, meningitis, and wound and bloodstream infections. These infections affect immunocompromised patients in the ICU, elders,

and neonates. HvKP causes similar infections but is additionally associated with liver infection, soft tissue infections, and endophthalmitis (**Figure 5**). Importantly, HvKP is connected with community-acquired infections as it can infect and cause disease in healthy people. Besides immunodeficiency, patients having diabetes, chronic liver disease, organ transplantation, dialysis, chemotherapy, and being treated with corticosteroids are more prone to acquiring nosocomial *Klebsiella* infection [204].

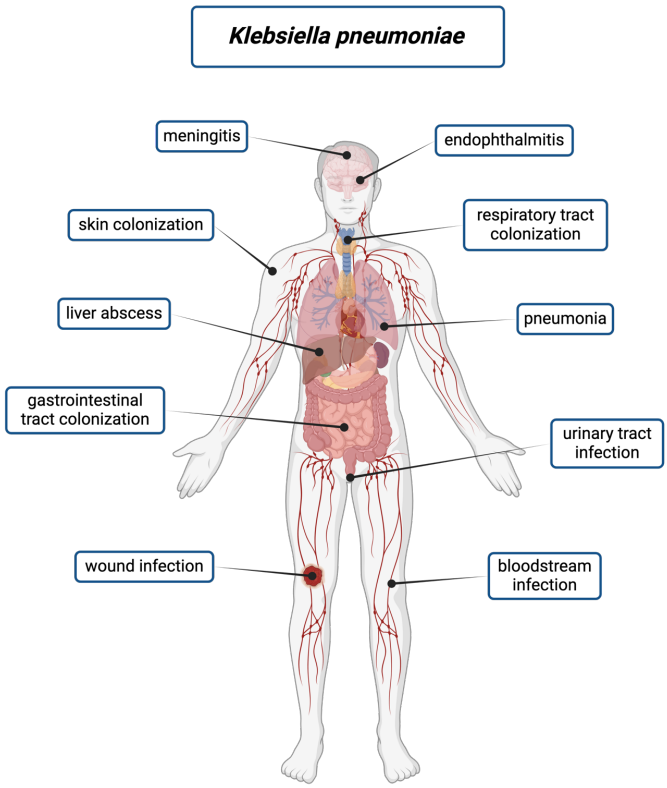

Figure 5. *K. pneumoniae*-mediated diseases and colonized surfaces.

Klebsiella-related infections are characterized by high lethality. A study from 2017 calculated that the general mortality of patients infected with carbapenem-resistant bacteria was 42.14%, while with carbapenem-sensitive was 21.16%. Based on the type of infection, the mortality rate for bloodstream infection was 54.30% and 13.52%,

while urinary tract infection was 48.9% and 43.13%, respectively. In Europe, the mortality rate was 50.06%, in North America 33.24%, in South America 46.71%, and in Asia 44.82% [205]. Bacteria are also spread globally. A meta-analysis from 2023 revealed that the global prevalence of carbapenem-resistant *K. pneumoniae* among patients was 28.69%. Prevalence in Western Europe was 42.05%, 14.29% in North America, and 66.04% in South Asia [206].

K. pneumoniae strains can be classified based on the sequence type (ST) defined by sequences of seven housekeeping genes (*mdh*, *infB*, *tonB*, *gapA*, *phoE*, *pgi*, and *rpoB*). CKP strains are associated with ST11, ST258, and ST512, while HvKP strains often belong to ST23, ST65, and ST86. However, new STs are being identified and gaining notoriety based on their virulence and AMR. Outbreaks are usually connected to the specific ST [207]. Interestingly, a report from China highlighted the occurrence of ST11 carbapenem-resistant CKP strains gaining hypervirulent phenotype by acquiring a virulence plasmid [208].

Virulence factors

K. pneumoniae produces several virulence factors that help during infections (**Figure 6**). HvKP strains are characterized by higher expression of virulence factors than the CKP strains as they grow thicker polysaccharidal capsules and secrete more siderophores.

General virulence

Polysaccharides capsule is one of the most important and studied virulence factors of *K. pneumoniae*. The pathogen produces around 80 capsule types, although even 160 different types are predicted to be possible. The capsule is a thick, approximately 160 nm layer protecting bacteria from the environment and during the infection from phagocytosis, antimicrobials, and lysis [209]. Encapsulated strains were also shown to induce lowered expression of TNFα and IL-6 and increased expression of IL-10. Capsule production can be upregulated by *rmpA*, *rmpA2*, *rcsA*, *rcsB*, and *magA* genes. Additionally, increased concentrations of glucose or iron can downregulate capsule production. CKP was shown to produce any identified K type, while HvKP K1 and K2 types are the most common and the most virulent [210].

Overproduction of the capsule is typically associated with the HvKP, which makes bacteria hypermucoviscous. This phenotype is commonly identified using a string test where a bacterial colony touched with an inoculation loop forms a viscous rope longer

than 5 mm. However, biomarkers such as *peg-344*, *iroB*, *iucA*, *rmpA*, and *rmpA2* might be more precise to distinguish HvKP and CKP [209].

Klebsiella produces several OMPs. OmpA plays a role in bacteria virulence, as the *ompA* mutants had decreased virulence in the mouse pneumonia model [211]. Mutants also were shown to trigger the secretion of IL-8 in epithelial cells and TNFα and IL-6 in mouse pneumonia. OmpA is also involved in cell adhesion. OmpK35 and OmpK36 are both involved in virulence, as shown in the mouse infection model, where mutants were less virulent than strains expressing both proteins [210].

Type 1 and type 3 fimbriae produced by bacteria are involved in adhesion and biofilm formation. Type 1 fimbriae are thin outer membrane structures consisting of the FimA domain and the adhesive FimH domain. They usually bind D-mannosylated glycoproteins. Type 3 fimbriae are helix-like structures made of the MrkA domain and MrkD binding domain. Type 3 fimbriae do not bind mannose, opposite to type 1. Both types are expressed in almost all *K. pneumoniae* isolates but are not produced in the planktonic form of bacteria [212]. Type 3 seems to be more critical in the biofilm formation. Interestingly, they are not required for gastrointestinal tract colonization but are essential for urinary tract colonization, probably due to catheter colonization. Type 3 is also involved in ventilator-associated pneumonia, probably mediated by bacteria in endotracheal tubes [210].

HvKP secretes colibactin and microcin E492, proteins involved in cytotoxicity. Colibactin is a toxin inducing DNA damage in host cells, as shown in a mouse model. Microcin E492 is a bacteriocin effective against bacteria of the *Enterobacteriaceae* family. However, microcin was shown to induce apoptosis in HeLa and Jurkat cell lines [213].

K. pneumoniae also secretes OMVs that might play a role in inflammation as they carry LPS [214]. They can also increase the spread of virulence factors and antibiotic resistance. Plasmids pLVPK and pK2044 have been characterized as carrying virulence genes, including capsule regulatory genes *rmpA* and *rmpA2* and siderophore-related gene clusters. OMVs contain plasmids, so their secretion might regulate the gene transfer [215].

Complement evasion

The bacterial capsule also mediates complement resistance, although the *K. pneumoniae* capsule is not considered a key factor. The capsule protects bacteria from the

complement-mediated lysis, being a barrier for MAC deposition. It can also decrease C3b opsonization as it might cover antigens for the complement activation [216]. It is speculated that the *K. pneumoniae* capsule might be sialylated, which slightly inhibits the activation [217].

LPS is the main structure involved in serum resistance. *Klebsiella* produces smooth LPS as it contains full-length O-antigen. Contrary to the K type, there are only nine O-antigen types, with O1 being the most common [210]. Smooth LPS is associated with complement resistance, as bacteria with rough LPS are complement-sensitive. Studies showed that rough LPS activated CP while smooth LPS activated AP. That would suggest that O-antigen protects bacteria from C1q or antibodies binding. Analyses of complement-resistant isolates showed lower deposition of C3b and MAC on bacterial surfaces. Therefore, LPS with O-antigen protects bacteria from complement deposition and lysis. Interestingly, OMVs carrying LPS on the surface might also be involved in the serum resistance, as it was shown that adding purified LPS to the complement-sensitive isolates protected them from MAC-mediated killing [216]. Interestingly, a mutation in the PhoQ kinase involved in LPS production was shown to increase the complement sensitivity [218].

OMPs are mentioned in serum resistance. Proteins Pal and LppA were shown to be involved in protecting against complement and phagocytosis. Mutant strains had disturbed the outer membrane, indicating their role in membrane stability. However, the exact mechanism has not yet been identified [219].

Secretion systems

T6SS has been identified in the hypervirulent *K. pneumoniae*. Bacteria use it to compete with other bacteria like *E. coli*. Interestingly, antibiotic stimulation may upregulate the expression of T6SS and influence the competition. *Klebsiella* was also shown to kill fungi in the experiments with *Candida albicans* and *Saccharomyces cerevisiae*. T6SS also played a role in the virulence against *G. mellonella*, as larvae survived better when infected with the mutant strain [220]. T6SS was also involved in the virulence against human cells when bacteria expressing the system caused mitochondrial fragmentation in A549 cells. T6SS was shown to trigger the release of Ca^{2+} from the endoplasmic reticulum, which triggered the production of ROS in mitochondria and consequently limited the activation of NF-κB. Moreover, T6SS was shown to be involved in the expression of type 1 fimbriae and adhesion and invasion into the Caco-2 cells.

Noteworthy, bacteria expressing T6SS were shown to carry more virulence-related genes, indicating that the system might be necessary for general virulence [221].

Survival mechanisms

K. pneumoniae secretes four siderophores important for iron scavenging. Their secretion is upregulated in HvKP compared to CKP. They are enterobactin transported into the cell via the FepA protein, yersiniabactin transported via YbtQ, salmochelin transported via IroN, and aerobactin transported via IutA. Their affinity to iron varies as enterobactin has the highest affinity, followed by yersiniabactin, salmochelin, and aerobactin, with the lowest affinity [210]. Enterobactin is a crucial iron uptake system as it is expressed ubiquitously in CKP and HvKP and helps bacteria during infection. Interestingly, the production of enterobactin can be inhibited by the host protein lipocalin-2, an antimicrobial molecule secreted by neutrophils. Yersiniabactin is heavily upregulated in HvKP and is not inhibited by lipocalin-2 but is affected by the transferrin in the blood. Thus, lung infection with *Klebsiella* producing only yersiniabactin is less problematic as bacteria cannot spread from the lungs. Salmochelin is an isoform of enterobactin and, as an isoform, cannot be inhibited by lipocalin-2. Salmochelin is involved in the nasopharynx colonization. Aerobactin seems important for HvKP as the mutant lacking aerobactin was less virulent in the mouse pneumonia model. Over 90% of HvKP produces yersiniabactin, salmochelin, and aerobactin, while they are secreted in less than 20% of CKP [210].

Quorum sensing in *Klebsiella* participates in producing fimbriae, capsules, LPS, biofilm, and iron acquisition systems. These factors might influence the AMR in *Klebsiella*. Interestingly, *Klebsiella* does not produce N-acyl homoserine lactones but can respond to them once secreted by other bacteria. Thanks to the LuxS synthase, bacteria produce furanosyl borate diesters called AI-2 that are secreted outside the cell. AI-2 molecules are taken up by bacteria and transported into the cytosol, where LsrK phosphorylates them to enhance the uptake. Once the optimal concentration of AI-2 is reached, the LsrR receptor is activated, and the signaling pathway is triggered, resulting in the protein expression [187].

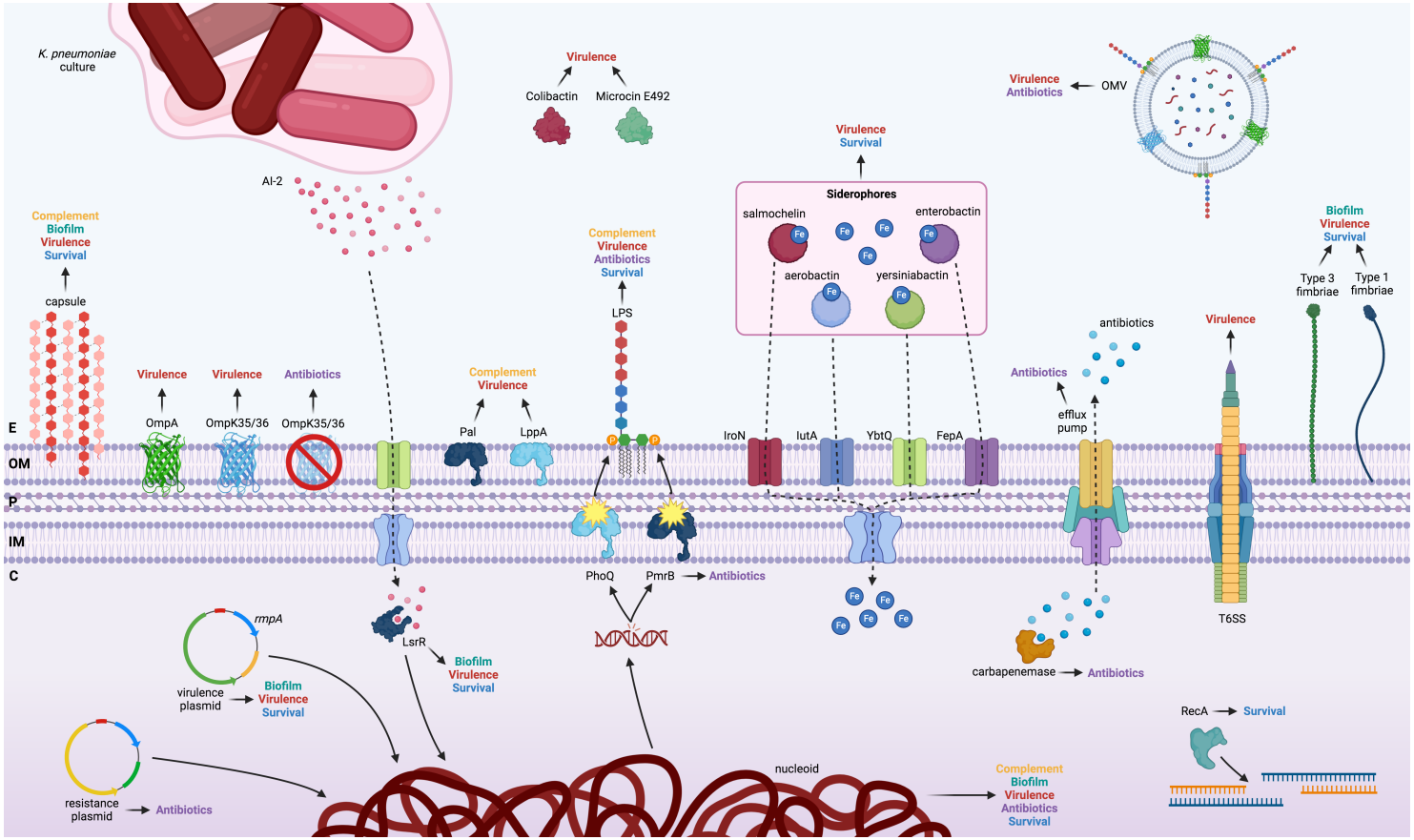

Figure 6. *K. pneumoniae* virulence factors involved in the complement resistance, biofilm formation, host virulence, antibiotic resistance, and survival (E – environment, OM – outer membrane, P – peptidoglycan, IM – inner membrane, C – cytoplasm).

Antibiotic resistance

Antibiotic resistance is the most problematic aspect of *Klebsiella*. Carbapenem-resistant CKP has gained significance in recent years. Worryingly, MDR bacteria are becoming XDR and even PDR, as we observed in isolates from patients in Ukraine [159]. Moreover, the problem is the spreading of AMR, as many enzymes, efflux pumps, and regulatory genes are transferred with plasmids. One study estimated that over 400 AMR genes were acquired by *K. pneumoniae*, almost double the number of genes in *E. coli* or *A. baumannii*. *Klebsiella* was also mentioned to carry, on average, more plasmids than other pathogens [222]. Although more evidence is required, *K. pneumoniae* may gain AMR through homologous recombination using RecA protein, which is common in gram-negative bacteria. Stimulation of *Klebsiella* with high doses of ciprofloxacin resulted in the SOS-mediated increase in resistance to rifampin [223].

Carbapenemases

K. pneumoniae expresses β-lactamases similarly to *A. baumannii*, but the most important clinically are carbapenemases. Carbapenemases can degrade most β-lactams, including carbapenems. As members of β-lactamases, they can be arranged according to the Ambler classification into A, B, C, and D classes. Noteworthy, C-type enzymes have been rarely reported. Class A includes IMI, SME, GES, and KPC enzymes. KPC enzymes are the most common worldwide and contain over 90 variants. KPCs are encoded on transferable plasmids, which have helped to spread them globally since their discovery in 1996. They display activity against all β-lactams. GES enzymes were first identified in 2000 in French Guiana and are also found in plasmids. Not all variants of GES enzymes have been shown to degrade carbapenems. Class B includes mainly NDM, VIM, and IMP enzymes, which can digest all β-lactams and carbapenems except for monobactams. They are located on plasmids and can be easily transferred between bacteria. NDM-1 was discovered in 2008 in India. Since then, it has become endemic in India, Pakistan, and Bangladesh and has spread worldwide. Over 20 types of NDM have been identified so far. VIM enzymes were first found in 1997 in Italy and are currently common in Southern Europe. Class D includes OXA enzymes, where OXA-48 is the most critical and prevalent. It has been first discovered in 2001 in Turkey. OXA-48 can also be easily spread on plasmids [224, 225].

Outer membrane proteins

Low expression of OmpK35 and OmpK36 was shown to increase the carbapenem resistance. However, the increase in resistance was minor, as shown in some studies. Probably the combination of OMP deletion and expression of β-lactamases/carbapenemases influenced the AMR [209].

Efflux pumps

The main efflux pumps in *K. pneumoniae* belong to the RND superfamily. AcrAB pump forms a complex that spans the whole cell wall and pumps out antimicrobials such as tetracyclines, chloramphenicol, quinolones, β-lactams, macrolides, and aminoglycosides. AcrAB is prevalent in *Klebsiella* isolates, and its overexpression can be stimulated by RamA and RarA regulators. OqxAB is another major RND pump that provides resistance against quinolones, tigecycline, nitrofurantoin, quinoxalines, and chloramphenicol. Interestingly, it is a plasmid-encoded pump, thus posing a threat of spreading AMR to other bacteria [226]. In one study, ST11 *K. pneumoniae* was found expressing pumps from five superfamilies: AcrAB, AcrD, KexD from RND, MacAB from ABC, KpnEF from SMR, KdeA from MATE, and EmrAB from MFS superfamily [227].

Colistin resistance

PDR of *K. pneumoniae* is currently the major problem that the healthcare system has to face as bacteria become resistant to polymyxins. Worryingly, the global colistin resistance in carbapenem-resistant *Klebsiella* increased from 2% to 9%. In Europe, the problematic situation is in Spain, Italy, and Greece, where the resistance reaches even 40% [228].

Colistin targets LPS by binding with negatively charged lipid A. Synthesis of LPS is regulated by two-component systems, PhoP/PhoQ and PmrA/PmrB. Therefore, mutations in these proteins lead to changes in lipid A charge, rendering bacteria resistant to colistin's antibacterial effect [218]. Interestingly, the regulatory gene *mgrB* affects the activation of the PhoP/PhoQ system. Thus, mutations of MgrB proteins also influence resistance. Noteworthy, alterations of the *mgrB* gene are considered the most common drivers of colistin resistance. The resistance can be exchanged using plasmid transfer with genes like *mcr*. The m*cr-1* gene was the first gene of colistin resistance shown to be spread on plasmids. Genes 1-10 have been identified, but *mcr-1* is the most common. The Mcr-1 protein modifies lipid A charge so colistin cannot bind

[229]. The influence of efflux pumps on colistin resistance is not evident. KpnEF pump is speculated to affect resistance as the mutation decreases the resistance. Moreover, the SMR superfamily that contains KpnEF is said to transport some antibiotics like colistin, ceftriaxone, erythromycin, and rifampicin. The AcrAB pump probably influences polymyxin resistance due to the pump's overexpression. A similar effect was observed when the efflux pump inhibitor was used. The bacterial capsule is speculated to affect the resistance as it might be a physical obstacle for colistin to bind LPS. However, the actual impact seems to be only minor [230].

Treatment options

With the increase in AMR, possible treatment options are limited. Monotherapy with colistin proved effective, but the rise of resistance mechanisms questions the idea of continuation. Therefore, a combination with other antimicrobials might be an option. The broad-spectrum antibiotics tigecycline and fosfomycin have been proposed to treat pneumonia and urinary tract infections. Their effectiveness was increased if used in combination with each other or with colistin. Several novel antimicrobials have recently been approved for therapy. A synthetic aminoglycoside – plazomicin, a siderophore-like cephalosporin – cefiderocol, and a synthetic tetracycline – eravacycline have been tested and showed promising in treating *K. pneumoniae* infections. Nevertheless, many cases of resistance against these antimicrobials have been reported. Additionally, combined therapies with β-lactam antibiotics and β-lactamase inhibitors have been suggested as a potential treatment option [228, 231].

An alternative option might be monoclonal antibodies against *Klebsiella* to increase the opsonization and phagocytosis. They might support the standard methods used during infections. Similarly, bacteriophage-based therapy was shown to be effective in animal models. As phages are gaining popularity, their use as therapeutic is still far from being a standard. They require more efficacy testing, and unfortunately, bacteria can develop resistance to them. A noteworthy idea is to use the fecal microbiota for transplantation. It might be collected from healthy donors and introduced to patients to replace their resistant microbiota. This process might work best in the hospital environment where immunocompromised patients are especially endangered [228]. Finally, developing vaccines against *K. pneumoniae* has been considered for many years. Bacterial capsules, LPS, O-antigen, fimbriae subunit proteins, OMPs, and OMVs have been used in

immunization. They were shown to be protective in animal models; thus, potential vaccine strategies exist. Nevertheless, more studies are required [232].

Streptococcus pyogenes

General information

Streptococcus pyogenes was first identified as a microorganism in 1874 by Austrian surgeon Theodor Billroth. He described bacteria forming chains of berries (*streptos*, meaning chain, and *kokkos*, meaning berry in Greek) [233]. In 1879, Louis Pasteur isolated the bacterium from a woman suffering from puerperal fever. He described that the pathogen was the reason causing the disease. The full name *Streptococcus pyogenes* was proposed in 1884 by Fredrich Julius Rosenbach when he studied bacteria isolated from the pus of the infected patient (*pyo*, meaning pus, and *genes*, meaning forming in Greek) [234].

Further differentiation of bacteria began in 1903 when Hugo Schottmuller introduced blood agar plates [235]. He observed hemolysis zones surrounding growing colonies. In 1919, James Howard Brown categorized these observations into three classes of hemolysis: α – very weak hemolysis, characterized by a greenish color caused by the reduction of hemoglobin to methemoglobin, β – full hemolysis caused by secreted virulence factors, and γ – no observed hemolysis [236, 237]. In 1933, Rebecca Lancefield further categorized bacteria based on their surface antigen composition [238].

Therefore, *S. pyogenes* can be classified as gram-positive, β-hemolytic, and Group A *Streptococcus* (GAS). Bacteria are encapsulated, non-motile, and form chains made of many cocci. GAS is an exclusively human pathogen that colonizes surfaces such as the nasopharynx and skin. It can be transmitted through saliva via direct contact or airborne droplets [234].

Clinical importance

Although GAS was isolated in the 19th century CE, the history of streptococcal diseases goes back to Hippocrates and the 4th century BC. GAS infections are associated with approximately 500,000 deaths yearly. Infections are usually acquired in hospitals, schools, homeless shelters, and care homes, where they can spread with saliva or direct contact. GAS can infect healthy people, but the most endangered are people with weakened immune systems or with chronic illnesses, the elderly, and children up to 15

years old. More infections are reported in developing countries with poor hygiene standards and poor healthcare systems. Nevertheless, an increase in infections has been recently reported in developed countries [239]. Noteworthy, the connection between poor hygiene and the risk of infection was observed as early as the 19th century by a Hungarian physician, Ignaz Semmelweis, working in a hospital in Vienna. He noted higher mortality among women giving birth in the wards where physicians did not wash their hands and spread diseases from autopsy rooms. He ordered all medical staff to clean their hands deeply and change bedsheets between patients to increase hygiene standards. His practices reduced mortality due to puerperal fever from 20% to less than 2% [234].

GAS causes many diseases, from mild pharyngitis and impetigo to life-threatening bloodstream infections and necrotizing fasciitis (**Figure** 7). Infections can also lead to chronic diseases such as rheumatic heart disease and glomerulonephritis [239, 240]. GAS infections are treated using antibiotics as the bacterium is not severely resistant. Only recently did the WHO include GAS in the AMR pathogen list when the macrolide-resistant GAS was included in the medium priority group [144].

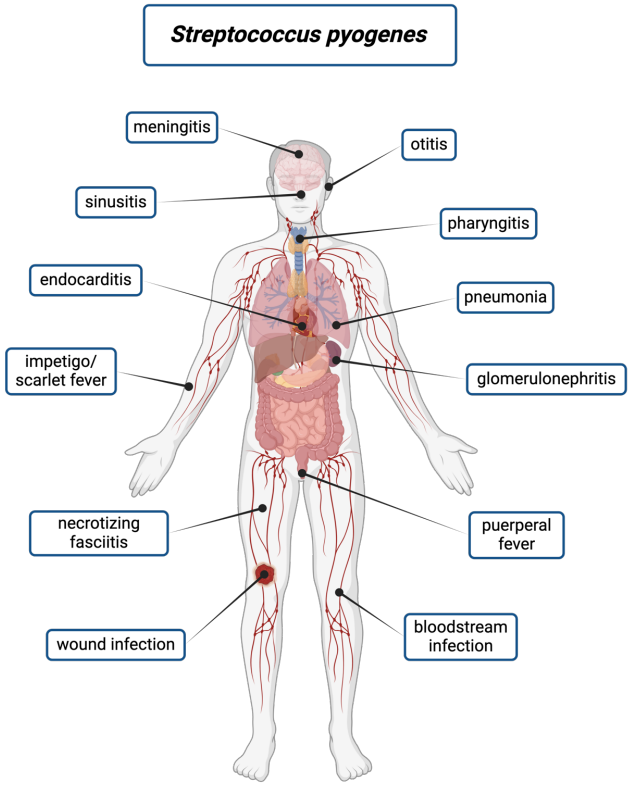

Figure 7. *S. pyogenes*-mediated diseases and colonized surfaces.

Virulence factors

S. pyogenes produces numerous surface-attached and secreted virulence factors (**Figure 8**).

General virulence

GAS capsule is a polymer made of glucuronic acid and *N*-acetylglucosamine. Production of the capsule is the highest during the exponential phase and is regulated by the *has* operon. Structurally, the GAS hyaluronic acid capsule is identical to human hyaluronic acid, thus helping the bacterium to hide from the host immune system. Capsule binds to the human hyaluronic acid receptor CD44, consequently allowing the bacterium to attach to host cells and colonize surfaces. Capsule production also

affects complement-mediated phagocytosis as the capsule dampens opsonization with C3 fragments. Capsule mutants were more susceptible to phagocytic killing [240, 241].

M protein encoded by the *emm* gene is the primary virulence factor in GAS. GAS classification is based on the M-serotypes; over 220 serotypes have been identified. M protein is a dimeric, coiled, fibrillar cell-wall protein that extends outwards approximately 60 nm. The C-terminus contains conserved among the GAS domain, while the N-terminus is hypervariable [242]. Due to gene duplications, GAS also produces M-like proteins such as H and Enn, which retain similar structures and functions as the M protein. Interestingly, protein H is expressed by approximately 30% of M1 strains. M proteins are crucial for GAS virulence as they bind albumin, fibrinogen, plasminogen, and immunoglobulins, thus contributing to the immune system evasion. They also evade complement by binding C4BP and FH. Complement inhibitors mediate the degradation of C3 fragments and reduce the opsonization of bacteria. M proteins are also involved in adhesion and colonization. Bacteria have been reported to interact with several cell receptors like CD46, but the interactions seem to be M-serotype specific [240, 243].

Lipoteichoic acid is a cell wall component involved in adhesion and biofilm formation. It participates in the first adhesion step, forming weak interactions with the host cell membrane. Further, other adhesins form more stable connections between cells. Pili are another cell wall structure involved in adhesion. These rod-shaped structures are essential in the attachment to human tissues and colonization of the human pharynx and skin [244].

SpeB is a secreted cysteine proteinase and a major virulence factor. SpeB was shown to be expressed in all GAS strains. The 40 kDa zymogen is cleaved into the 28 kDa active enzyme, which can cleave over 200 proteins such as cytokines, chemokines, complement proteins, immunoglobulins, and ECM proteins. In laboratory conditions, SpeB was shown to cleave the Fc and Fab domains of IgG. Additionally, the enzyme rescues the M1 strain intracellularly by cleaving p62, NDP52, and NBR1 proteins involved in the autophagy. It can induce inflammation by activating IL-1β and gasdermin A [245]. SpeB greatly affects the complement proteins as it can disrupt all pathways. Proteinase was proven to cleave C2, C3, C4, C5a, C6, C7, C8, and C9, thus disturbing functions of the whole cascade. It can also cleave complement regulators C1-INH and properdin [246].

Besides SpeB, GAS secretes other immunoglobulin-degrading proteins. IdeS/Mac-1 is a cysteine proteinase discovered independently by two groups, hence the name. This 35 kDa endopeptidase targets the lower region of the heavy chain in the deposited IgG, thus generating $F(ab')_2$ and Fc fragments. Degradation protects bacteria from complement activation and Fc receptor-mediated phagocytosis. Some GAS serotypes also express an allelic variant called Mac-2, probably contributing to IdeS/Mac-1 functions [247]. EndoS is a 108 kDa highly conserved secreted endoglycosidase. It targets glycan moiety present on the heavy chain of IgG. Cleavage mediates complement and phagocytosis evasion as the glycosylation at Asn297 is crucial for interacting with receptors. EndoS2 is a smaller, 95 kDa version of EndoS identified in the serotype M49. EndoS2 is somewhat more active than EndoS as it has a broader spectrum and targets bigger glycans [245]. GAS also secretes the SibA protein that was shown to bind the Fc and Fab domains of IgA, IgG, and IgM. The role of this 45 kDa protein is not clear but is speculated to be involved in virulence due to the ability to acquire immunoglobulins [248].

Streptolysins SLS and SLO are important toxins involved in virulence against host cells. SLS is a very small 2.7 kDa secreted toxin responsible for the lysis of erythrocytes and β-hemolysis specific to GAS. SLS lyses also include lymphocytes, neutrophils, macrophages, and organelles. The toxin is important in necrotizing fasciitis as it is upregulated during the invasive infection, and the strains lacking SLS are less virulent. Moreover, SLS was shown to stimulate pain-sensing receptors to release neuropeptides, reducing neutrophil recruitment and thus increasing GAS survival [249]. SLO is a 69 kDa exotoxin, causing lysis of the host cells. SLO attaches to the cell surface and forms a prepore. Further, conformational changes in the SLO monomers create large β-barrel pores. The toxicity can be enhanced by NADase, which can be translocated into the cytosol of host cells via SLO pores. Additionally, SLO activates inflammasome, IL-1β release, and apoptosis of neutrophils and macrophages. Interestingly, SLO and NADase were shown to inhibit the release of IL-1β and IL-8 by stimulating ubiquitination and degrading the Golgi apparatus, respectively. SLO and NADase also regulate GAS survival intracellularly. They were shown to mediate evasion from autophagy and endocytosis [249].

SpyCEP is a serine protease expressed as a zymogen cleaved into 20 and 150 kDa fragments to be activated. It is attached to the cell wall via the sortase and released into culture during the stationary growth phase. Once activated, it hydrolyzes CXC chemokines such as CXCL1, CXCL2, CXCL6, and CXCL8, providing evasion from

neutrophils. It was also shown to degrade LL-37 and dampen the LL-37-mediated signaling [250].

GAS also expresses DNases Sda1 and SpnA. Sda1 is a secreted enzyme degrading NETs, thus promoting the bacterium's survival. Intriguingly, Sda1 degrades CpG-rich DNA, dampening TLR9-mediated signaling and production of TNFα and IFNγ [251]. SpnA is a cell well-attached DNase also degrading NETs. SpnA activity depends on the presence of Ca^{2+} and Mg^{2+} ions [252].

Streptokinase is a secreted plasminogen-activating protein. It regulates the activation of plasminogen into the active plasmin. Plasmin can degrade host ECM proteins, fibrin clots, and connective tissues, enhancing the spread of GAS during infection. Plasmin also degrades LL-37, C3b, and histones, contributing to the pathogen's survival. Streptokinase activates the plasminogen by forming a complex with the zymogen and non-enzymatically changing the conformation of the protein [253]. Some GAS serotypes produce hyaluronidase, which can degrade hyaluronic acid and promote the spread of bacteria. Interestingly, M4 and M22 were shown to lose the ability to create a hyaluronic acid capsule but could express hyaluronidase. Due to the lack of capsules, these serotypes possibly employed alternative methods to remain virulent [254].

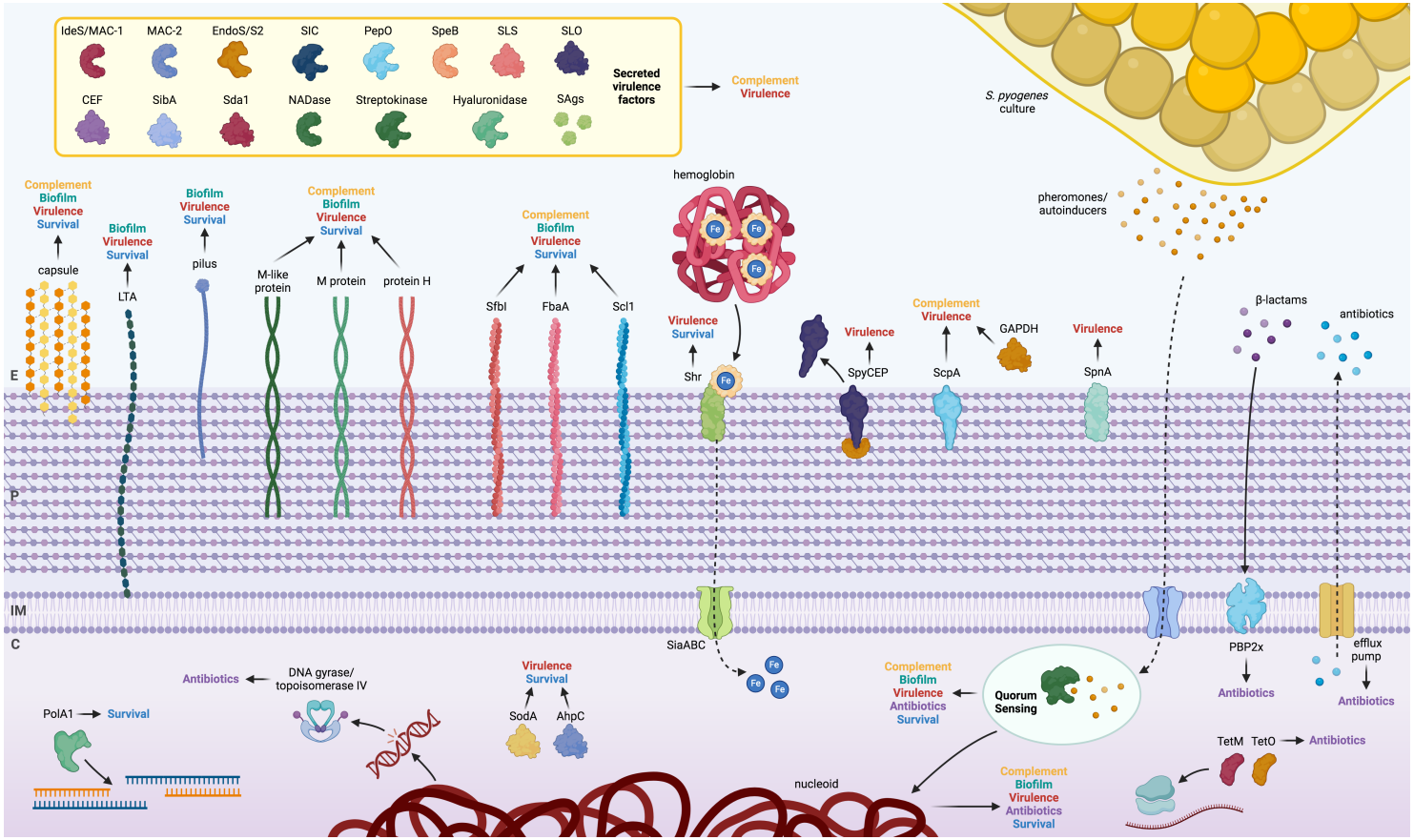

Figure 8. *S. pyogenes* virulence factors involved in the complement resistance, biofilm formation, host virulence, antibiotic resistance, and survival (E – environment, P – peptidoglycan, IM – inner membrane, C – cytoplasm).

Superantigens are 25 kDa secreted exotoxins. Eleven different genes encoding superantigens have been identified in GAS. They can bind simultaneously to MHC class II receptors on the antigen-presenting cells and T cell receptors on T cells in an atypical manner. They interact with MHC class II receptors outside the typical domain for normal antigens. Nevertheless, they activate T cells and stimulate the release of pro-inflammatory cytokines such as TNFα, TNFβ, IL-2, IL-6, and IFNγ. Interestingly, pre-exposition with superantigens primed human monocytes to release more cytokines when cells were exposed to LPS. Higher expression of TLR4 in cells stimulated by superantigens was suggested as an explanation for this phenomenon. Overstimulation with superantigens leads to severe inflammation and contributes to diseases such as toxic shock syndrome and necrotizing fasciitis [255].

Complement evasion

As mentioned earlier, M and M-like proteins play a role in complement evasion by acquiring C4BP and FH. Besides M proteins, GAS produces other cell-wall proteins, FbaA, Scl1, and Sfb1, that bind complement proteins. FbaA is a 40.5 kDa fibronectin-binding protein that also can acquire FH. FbaA was shown to be involved in adhesion and pathogenesis as the *fbaA* mutant strain was less virulent in the mouse animal model [256]. Similarly, Scl1 is a 44.5 kDa surface protein that binds FH and FHR-1 proteins. Scl1, expressed in all GAS strains, binds FH via CCPs 18-20 [257]. Sfb1 is involved in the invasion of epithelial cells and evasion from complement. The protein binds fibronectin and is speculated to protect the bacterium from C3b deposition [258].

C5a is degraded by a cell wall-attached serine endopeptidase ScpA. This 130 kDa enzyme targets C5a, reducing its ability to attract and activate phagocytic cells, thus protecting the bacterium from phagocytosis. The *scpA* mutant was efficiently removed from the lungs in a mouse infection model [259]. Remarkably, ScpA was shown to cleave C3 and C3a. The cleavage of C3 generated an impaired C3b, while C3a degradation reduced neutrophil recruitment. ScpA can be released from the cell wall by SpeB, thus degrading complement proteins in the environment. Noteworthy, ScpA can be supported by the GAPDH protein. GAPDH is generally a cytosolic protein, but on the bacterial surface, it can bind C5a, affecting its functions [259].

PepO, CEF, and SIC are virulence factors that target the complement cascade's first and last steps. PepO protein is a secreted endopeptidase that binds C1q and prevents interaction between C1q and IgG in conditions with low pH, similar to inflammation.

The mutant lacking PepO protein had decreased survival in the human whole blood and the *G. mellonella* model [260]. CEF is a newly discovered protein that binds C1s, C1r, C3, and C5, meaning it can theoretically inhibit all complement pathways. The expression of CEF in *Lactococcus lactis* resulted in the gain of function and improved survival in the whole human blood [261]. SIC is a 31 kDa MAC inhibitor. It binds C5b-7 and C5b-8, preventing C9 polymerization and MAC formation. Noteworthy, GAS is a gram-positive bacterium; therefore, peptidoglycan should protect from MAC deposition. Thus, it is interesting that GAS produces MAC-inhibitory protein [262].

Survival mechanisms

The acquisition of iron is crucial for GAS survival during the infection. GAS can obtain iron from hemoglobin, myoglobin, and haptoglobin-hemoglobin complex using the SiaABC transporter. Noteworthy, heme is an essential source of iron as the bacterium cannot utilize transferrin or lactoferrin. The acquisition is mediated by the surface-attached Shr protein that binds hemoglobin, myoglobin, and the haptoglobin-hemoglobin complex [180].

GAS produces several proteins that are involved in the resistance to ROS. SodA is a manganese-dependent dismutase that converts $O_2^{\bullet-}$ into H_2O_2 and O_2. SodA can be secreted extracellularly to protect bacteria from ROS produced by the host immune cells. The expression of SodA is upregulated in the presence of superoxide. AhpC is a NADH-dependent peroxidase degrading H_2O_2. AhpC production is directly upregulated during oxidative stress. GAS *ahpC* mutants showed that AhpC is essential in the survival and virulence of the pathogen. Also, DNA polymerase PolA1 is involved in the resistance to H_2O_2, as demonstrated by the *polA1* mutant that became sensitive to H_2O_2. PolA1 is additionally vital in DNA repair. A mutant for PolA1 had significantly more damaged DNA caused by UV light or ciprofloxacin treatment [263].

Quorum sensing is involved in GAS in the production of biofilm, expression of virulence factors, and conjugation. Four systems identified in the bacterium regulate protein expression upon the activation with secreted inducers [264]. The first system described in GAS is the Sil system, activated by the SilCR pheromones and involved in the colonization and general virulence of the bacterium. The second system contains proteins RopB, Rgg2, Rgg3, and ComR. RopB regulates the expression of many genes, including SpeB toxin. Rgg2 and Rgg3, activated by SHP pheromones, are responsible for biofilm formation and lysozyme resistance. ComR is involved in the horizontal gene transfer and is activated by XIP pheromones. Third is the lantibiotic regulatory system,

which helps the bacterium establish the infection. GAS produces antimicrobial peptides bacteriocins, which help in competition with other bacteria. Lantibiotics can form pores in the membrane, inhibit cell wall synthesis, or inhibit enzymes. They also function as pheromones and stimulate quorum sensing. Streptin, streptococcin, and salivaracin A are lantibiotics found in GAS. Finally, GAS expresses the LuxS/AI-2 system. LuxS produces autoinducers AI-2, which are released into the environment and used to communicate between bacteria. In the cytosol, AI-2 molecules bind LsrR and stimulate protein expression. In GAS, this system is involved in the biofilm formation, synthesis of the capsule, and expression of virulence factors such as SLS, M proteins, and SpeB [264].

Antibiotic resistance

Interestingly, GAS did not acquire significant and worrying AMR. Nevertheless, GAS is resistant to a few antibiotics.

β-lactams

β-lactams interact with penicillin-binding proteins, disrupting the synthesis of peptidoglycan. A recent report from the US identified resistance to β-lactams in two GAS isolates carrying *emm43.4*. The study showed a single-point mutation in the PBP2x protein. Isolates had a substitution T553K, which made them significantly less sensitive to the effect of ampicillin and cefotaxime. These findings highlighted the possibility of GAS becoming resistant to β-lactams [265].

Macrolides

GAS is mainly associated with macrolide resistance, as reported by WHO. The first resistant isolates were identified in 1968 in the US. Currently, the macrolide resistance in the US remains at around 5%. Southern Europe reported a higher resistance rate at approximately 20%. However, the resistance rate in Asia is much higher, especially in China, reaching even 90-95% [266]. The bacterium can resist macrolides, lincosamides, and streptogramin B due to the erythromycin resistance methylase genes *ermB*, *ermTR*, and *ermT*. Genes *ermB* and *ermTR* are localized on chromosomes, while *ermT* is found in a plasmid. GAS becomes resistant thanks to the methylation of 23S rRNA. Macrolides target ribosomal subunit 50S; thus, the methylation of rRNA changes the binding site, rendering bacteria resistant. GAS can also actively pump out

14- and 15-membered macrolides thanks to the efflux pumps encoded by *mefA* and *msrD* genes [240].

Tetracyclines

Resistance to tetracycline is mediated via the *tetM*, *tetO*, *tetK*, and *tetL* genes. When tetracycline tries to bind the ribosomal subunit 30S, TetM and TetO displace it. Simultaneously, TetK and TetL efflux pumps remove the antibiotic from the cytosol. Interestingly, tigecycline, related to tetracycline, has a similar mechanism of action, but GAS did not develop resistance to this antimicrobial [266].

Fluoroquinolones

Mutations in *parC* and *gyrA* genes mediate resistance to fluoroquinolones. Genes *parC* encodes topoisomerase IV, while *gyrA* encodes DNA gyrase. Therefore, mutations in these proteins lowered the binding affinity of fluoroquinolones [240].

Treatment options

Luckily, GAS infections are treatable with antibiotics such as β-lactams. However, the increase in macrolide resistance and the sporadic occurrence of β-lactam-resistant isolates underscore the possibility of further resistance emergence among clinical streptococci. As mentioned in the literature, good hygiene helps to prevent the spread of bacteria in the community. Nevertheless, vaccination against GAS would be the best option to limit infections. Unfortunately, despite decades of research, no vaccine has been approved for commercial use. Already in the 1920s, whole bacteria and fragments of M proteins have been used for vaccination. Most of the proposed vaccines targeted M proteins, but the high number of serotypes impeded the development. In the last 20 years, only four vaccines entered clinical trials, and all four were M-based. The most recent was StreptAnova, a 30-valent vaccine formulated based on the 30 most important M serotypes. StreptAnova was an enhanced version of the previous 26-valent StreptAvax vaccine. Based on the phase I clinical trials from 2020, StreptAnova vaccination resulted in the antibody production against 25/31 antigens. Another approach is to produce a non-M protein vaccine. Vaccines such as VAX-A1, Combo#5, and Spy7 are formulated based on the virulence factors expressed by GAS. They include proteins such as SLO, SpyCEP, and ScpA combined with an aluminum adjuvant. In the mouse animal models, these vaccines stimulated an immune response against GAS [240, 267].

Methodology

Bacterial cultures

Acinetobacter spp.

Isolates were routinely grown on blood agar plates, providing necessary nutrients for bacterial growth. Bacteria were streaked onto agar plates from glycerol stocks. The plates were incubated overnight at 37 °C. The following day, bacteria were subcultured onto fresh agar plates for three to five hours of incubation. During the screening study, we noticed that some strains grew slightly slower than others, with *A. baumannii* growing the fastest. We decided to incubate subcultured bacteria for five hours to give them time to grow. Once we focused only on *A. baumannii*, we reduced the incubation time to three hours. We noted that *Acinetobacter* spp. grew equally well at 30 °C and 37 °C. Interestingly, *A. johnsonii* isolates preferred to grow at 30 °C.

Capsule mutants were first prepared in brain heart infusion supplemented with necessary antibiotics: 307.30 with 50 μg/mL kanamycin; 307.30/pNLAC1::*ptk* with 50 μg/mL kanamycin and 200 μg/ml carbenicillin. However, we observed that mutants grown on blood agar plates retained their phenotype also without antibiotics.

Klebsiella pneumoniae

Isolates were grown under conditions similar to those used for *Acinetobacter* isolates. They were streaked and incubated on blood agar plates at 37 °C, first overnight and then for three hours the next day.

Streptococcus pyogenes

S. pyogenes, on the contrary, was grown in liquid using Todd Hewitt broth. The broth was inoculated with bacteria and incubated overnight without shaking at 37 °C in the presence of 5% CO_2. The next day, bacteria were diluted to OD_{600} = 0.1 in the fresh medium and incubated under the same conditions to reach the exponential growth phase with OD_{600} = 0.3-0.4. Todd Hewitt's broth is a nutritious medium specialized

for the growth of Group A Streptococci. To grow the wild-type strains, we used the medium without antibiotics. Mutant cultures were supplemented with necessary antibiotics: MC25 (lacking M1 protein) – 150 μg/ml kanamycin; BM27.6 (lacking protein H and M1) – 1 μg/ml erythromycin; BMJ71 (lacking proteins H, M1, SIC, and C5a peptidase) – 5 μg/ml tetracycline.

Acinetobacter and *Klebsiella* have been grown on agar plates, but when necessary, we have grown them in liquid culture using lysogeny broth or brain heart infusion medium. Lysogeny broth is a standard laboratory medium suitable for most bacteria. At the same time, brain heart infusion is a highly nutritious medium preferred by pathogenic bacteria, providing more nutrients than lysogeny broth.

The overnight incubation with additional subculturing allowed us to refresh cultures after overnight incubation to have bacteria in their exponential growth phase. This allowed us always to have bacteria in their optimal state for the experiments.

SYTOX Green assay

To measure the complement-mediated lysis of bacteria, we used the SYTOX Green assay. Bacteria were mixed with 30% human serum to activate complement and deposit MAC. The serum was supplemented with either nisin A or *Ornithodoros moubata* complement inhibitor (OmCI). Nisin A is a small peptide that can pass through the MAC pores and enhance bacterial killing [100]. Thus, we used it as a positive control for MAC-mediated pore formation in the bacterial outer membrane. OmCI, on the other hand, is a C5 cleavage inhibitor derived from the saliva of a thick *Ornithodoros moubata*; thus, we used it as a negative control [268]. Additionally, samples were supplemented with SYTOX Green dye, which stained bacterial nucleic acid, acting as a cell death indicator. We monitored the bacterial killing for two hours at 37 °C using a BioTek Cytation5 plate reader. We measured the increase in fluorescence of the SYTOX Green every 150 seconds over two hours (**Figure 9**).

Our collaborators have used this method extensively to measure the killing of *E. coli* and *K. pneumoniae* [100]. The standard procedure for assessing serum bactericidal activity is the tedious plating of bacteria and counting colony-forming units. With the SYTOX method, we can significantly simplify the experiment. The main advantage of this method is that we obtain results in just three hours compared to overnight incubation of agar plates. Of course, the incubation time can be extended to overnight incubation if necessary. Since we prepare the entire experiment in a 96-well plate, we

can simultaneously screen many isolates or test many conditions using different serum concentrations, antimicrobials, antibiotics, etc. Cytation5 allows measuring two distinct outcomes, so we can include, together with SYTOX, optical density measurements to monitor bacterial growth.

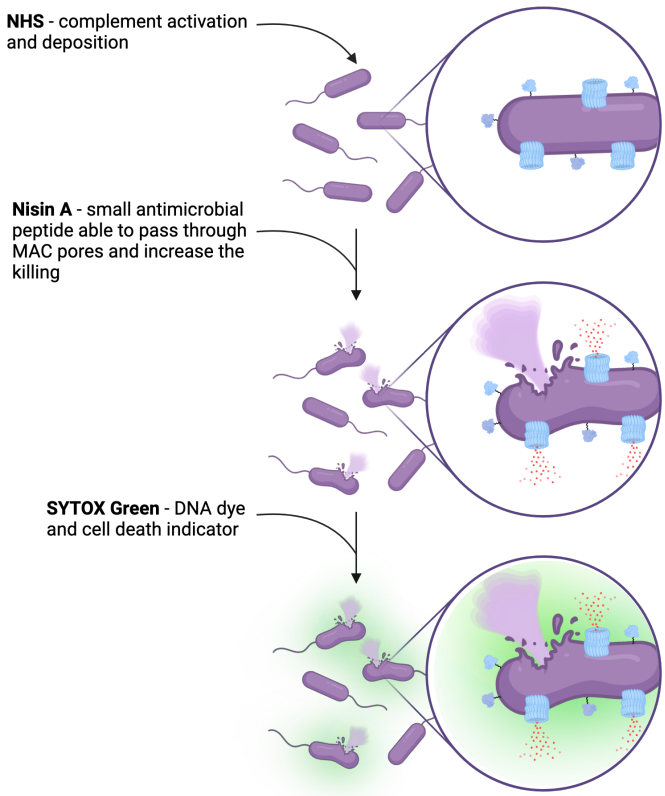

Figure 9. Overview of the SYTOX Green Assay.

When combining the SYTOX Green assay with the plating method, we must consider that the SYTOX method is more sensitive than plating. We may measure a significant SYTOX fluorescence, indicating bacterial killing, but observe no difference in the colony numbers. These two methods must be optimized explicitly to be used together.

Galleria mellonella infection model

To study the virulence of *A. baumannii* and *K. pneumoniae in vivo,* we used the *Galleria mellonella* infection model. Larvae were separated into fives in separate Petri dishes. Each fives was inoculated with bacteria at 10^8, 10^7, or 10^6 CFU/mL. For negative infection control, we used PBS buffer. We also included untreated larvae to monitor their condition during the incubation process. Larvae were injected with 10 µL of bacterial culture or PBS, returned to dishes, and incubated at 37 °C for five days (120 hours). Their survival was monitored twice daily, at 8 am and 8 pm. We monitored the melanization and movement of the larvae after the stimuli (**Figure 10**).

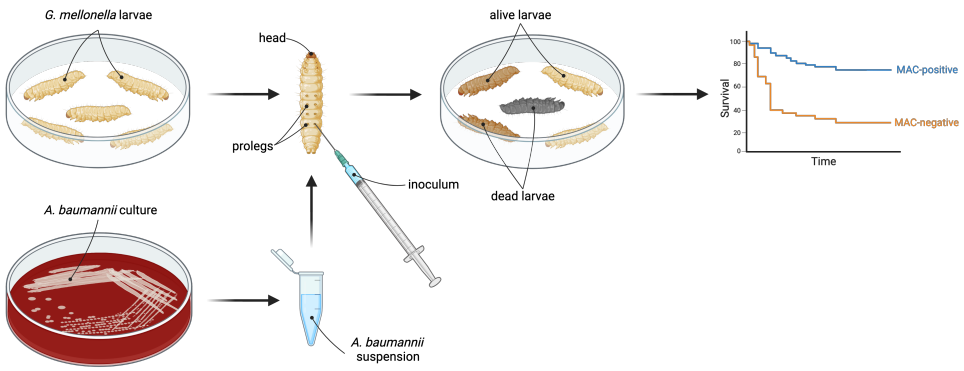

Figure 10. Overview of the *Galleria mellonella* infection model.

In vivo infection models are routinely performed using mouse models, as they mimic the human immune response well. *In vivo* experiments have an advantage over *in vitro* tests since bacteria behave differently inside the host, often presenting higher virulence. However, we must consider ethical aspects and minimize animal suffering when using vertebrates. Therefore, the 3Rs rule – Replace, Reduce, and Refine – has been recommended. Additionally, mouse models require permits, specialized equipment, and training and are time-consuming. Hence, invertebrates, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Galleria mellonella*, are often used. *Galleria* has been extensively tested in *A. baumannii* experiments [269].

G. mellonella, called the greater wax moth, belongs to the family *Pyralidae* of the order *Lepidoptera*. *Galleria* has four life stages, of which larvae are the most important as a model. Their advantage is surviving the incubation at 37 °C, making them perfect hosts

for bacterial growth. They are easy to maintain, inexpensive, and require no ethical permits; thus, they are ideal for screening experiments. As insects, they lack an adaptive immune system, but their innate immune system shares similarities with mammalian immunity [270, 271].

Hemocytes, an analog of the human neutrophils, are typically found in hemolymph, an analog of the human bloodstream. Six types of hematocytes have been identified, which are involved in clotting, encapsulation, phagocytosis, nodulation, and melanization. They have been shown to produce ROS and secrete molecules involved in the humoral response. Noteworthy, *Galleria* produces complement-like opsonins participating in phagocytosis. Apolipophorin-III can induce the production of ROS and antimicrobial peptides. In other insects, peptidoglycan recognition proteins (PRGPs) were shown to cleave peptidoglycan, while cationic protein 8 (GmCP8) and hemolin were shown to bind LPS or lipoteichoic acid. However, their functions in *Galleria* have yet to be discovered. Additionally, the production of lysozyme by *Galleria* has been documented [270, 272].

Melanization develops gradually during the infection and can be used to monitor survival since the complete melanization of larvae indicates death. The process occurs during prophenoloxidase activation. During infection, the proenzyme is released from hemocytes and activated by a cascade of serine proteases. Active phenoloxidase converts phenolic compounds to quinones, which are further converted to melanin. Serpins tightly control enzyme activation, as the products, quinones and free radicals, are cytotoxic to larvae. Melanin deposition is responsible for the black spots and general blackening in the infected worms. Noteworthy, apolipophorin-III was shown to stimulate phenoloxidase activation [270, 272].

Unfortunately, the infection protocols are not standardized. Furthermore, official suppliers who could provide high-quality larvae for experiments are absent. Larvae usually have to be obtained from animal shops, which cannot guarantee regulated conditions. Larvae exposed to bacteria prior to experiments may react differently than they should have in a situation of regulated production [271].

FH-IgG fusion proteins

We used the fusion protein to overcome the complement evasion mechanism in *Streptococcus pyogenes*. Proteins comprised CCPs of human FH and Fc domain of IgG (**Figure 11**). We have tested proteins with CCPs 6-7 and 18-20, as human pathogens

use these domains to bind FH – *S. pyogenes* is known to bind CCPs 6-7 primarily [147]. Proteins were expressed in the floating FreeStyle CHO cells and secreted into the medium, which we later collected. Next, proteins were isolated from the medium using the Protein A/G column. We tested their capacity to activate complement and protect transgenic mice during sepsis.

The principle behind fusion proteins is their ability to bind to bacteria using FH domains and displace FH that bacteria have acquired from serum. Once the proteins bind to the surface, they will expose Fc domains recognized by the C1 complex to activate CP and Fc receptors on phagocytic cells to ingest bacteria. Therefore, the serum FH will no longer protect bacteria from opsonization and phagocytosis. In the study, we tested constructs with human and mouse IgG since *S. pyogenes* binds human but not mouse immunoglobulins. Comparing both constructs, we concluded that the FH6-7/hFc protein binds bacteria via FH and Fc domains, while the FH18-20 construct binds only via Fc domains.

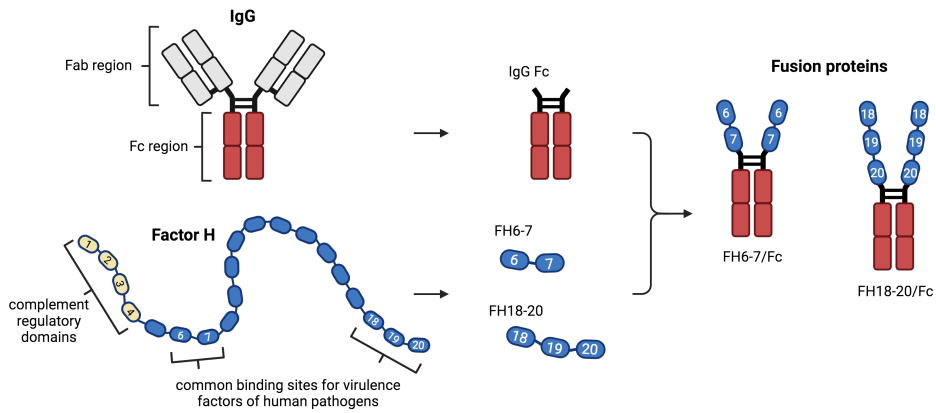

Figure 11. Schematic representation of FH-IgG fusion protein structures.

In this study, we used gram-positive *S. pyogenes* as a model. However, these fusion proteins were also effective against gram-negative bacteria, such as *Neisseria meningitis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* [273-275]. Noteworthy, fusion proteins from our study contained the IgG1 subclass, while our collaborators recently showed that switching to the IgG3 subclass could improve the complement-mediated killing of *N. gonorrhoeae* [276].

Human pathogens are also known to acquire another complement inhibitor, C4BP. Therefore, fusion proteins containing C4BP domains have also been constructed. Proteins consisting of CCPs 1-2 and IgM domain displaced C4BP and increased the killing of *N. gonorrhoeae* [277]. Interestingly, proteins also increased the killing of *M. catarrhalis* but without displacing C4BP from the bacterial surface [278]. Taken together, these findings suggest that fusion proteins may be used as therapeutics against pathogens. By counteracting their virulence mechanisms, the proteins enhance opsonization and complement-mediated killing.

Project aims

The general aim of the project was to elucidate the mechanisms of complement evasion in human pathogenic bacteria. The complement system is a crucial pathway in the defense against bacteria, and it is well known that pathogens evade complement by utilizing different strategies.

We investigated three significant human pathogens – *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes* – focusing on their ability to evade complement.

Therefore, the aim of each paper was:

Paper I

To screen clinical isolates of *Acinetobacter* spp. isolated from patients in Sweden and Northern Ireland, focusing on complement activation in the presence of bacteria.

Paper II

To characterize the MAC resistance and possibly describe the evasion mechanism of *A. baumannii* isolates.

Paper III

To examine clinical isolates of *K. pneumoniae* isolated from patients and war victims in Ukraine, focusing on their antibiotic resistance and complement resistance.

Paper IV

To evaluate the efficacy of FH-IgG fusion proteins in overcoming the complement evasion mechanism in *S. pyogenes*.

Present investigations

Paper I

Clinical isolates of *Acinetobacter* spp. are highly serum-resistant despite efficient recognition by the complement system.

Background

Bacteria of the genus *Acinetobacter* are known for causing severe nosocomial infections, predominantly in immunocompromised individuals. Given that the complement system plays a crucial role in the defense against microbial infections, it is vital to improve our understanding of the evasion strategies employed by *Acinetobacter* spp.

In this study, we screened 50 different clinical isolates of *Acinetobacter* spp. and measured their resistance to human serum. Bacteria were isolated from hospitalized patients in Belfast, Northern Ireland, and Lund, Sweden.

Findings

We observed that bacteria survived the incubation in 30% human serum. Some isolates had reduced survival, but none was entirely killed. Interestingly, four selected isolates endured a three-hour treatment with 50% serum, an equivalent of whole blood concentration. Remarkably, the same four isolates persisted in human blood, containing the complement system and phagocytic cells. Our pooled human serum contained IgG and IgM antibodies, which recognized *Acinetobacter* isolates, probably detecting common antigens in the gram-negative bacteria.

The AP was the main complement pathway activated on the bacterial surface, as the C4b deposition was detected only on some isolates. Noteworthy, we did not measure significant amounts of C1q, indicating transient binding of the protein. Although the AP was activated, we noticed a few isolates with lower deposition of C3b and C5b. As a result, less MAC was deposited on the bacterial surface, suggesting a possible complement evasion mechanism.

One of the mechanisms in *Acinetobacter* is the polysaccharide capsule that forms a barrier around the cell. All clinical isolates produced capsules of varying thickness, as we observed using a staining method. The importance of the capsule was demonstrated using the mutant strain. This strain became highly sensitive compared to the wild-type. Noteworthy, the capsule expression changed the activation pattern of the bacterium, as the wild-type activated LP while the mutant activated CP.

Our data showed a remarkable serum resistance in the tested isolates. We noted some isolates worth studying, as they deposited significantly less MAC. This indicated the presence of a complement evasion strategy that we wanted to elucidate.

Paper II

Acinetobacter baumannii **clinical isolates resist complement-mediated lysis by inhibiting the complement cascade and improperly depositing MAC.**

Background

As a gram-negative bacterium, *Acinetobacter* should be sensitive to the MAC-mediated lysis. Our previous findings showed complement resistance and, interestingly, decreased deposition of MAC in some isolates.

In this study, we continued analyzing the complement evasion of bacteria, focusing on MAC evasion. We studied only *A. baumannii*, as it is the most clinically relevant strain.

Findings

Using flow cytometry analyses, we observed MAC deposition on eleven isolates, while five lacked a significant amount of the complex. Interestingly, trypsinization of MAC on the MAC-positive isolates reduced the presence of the complex by approximately 80%. This indicated that MAC was improperly inserted into the outer membrane but only loosely attached to the surface. In the case of MAC-negative isolates, increasing amounts of serum did not increase MAC deposition. These observations were confirmed using C9-depleted serum supplemented with wild-type and mutant C9 proteins. Additionally, MAC-negative isolates caused less sMAC formation in the fluid phase.

Moreover, we tested bacterial MAC resistance using 30% serum and the antimicrobial peptide nisin A. Nisin A, being a small peptide, can pass through the MAC-mediated pores and increase the killing, which is further detected by a cell death marker, SYTOX Green. We did not observe any increase in the killing of MAC-negative isolates, as they were entirely resistant to serum. Interestingly, a slight increase in the killing in the presence of nisin was observed in the MAC-positive isolates, indicating that some cells were affected by MAC.

Next, we analyzed earlier steps of the complement cascade using flow cytometry and western blotting. We observed decreased C3b, FB, and C5b deposition on MAC-negative isolates compared to MAC-positive bacteria, explaining the absence of MAC. Noteworthy, properdin deposition was also reduced, suggesting an inhibition of the early step of AP.

The *Galleria mellonella* infection model revealed that MAC-negative isolates were significantly more virulent than MAC-positive bacteria, killing more larvae. We observed higher virulence at every tested concentration of bacteria.

Finally, we sequenced the genomes of bacteria, looking for differences in virulence factors. The data showed no quorum sensing and type VI secretion system gene expression in most MAC-positive isolates. MAC-negative isolates expressed both systems, which may explain an elevated virulence against *Galleria* larvae. Noteworthy, three out of five MAC-negative isolates expressed the same K171 capsule type, which may be involved in the MAC resistance.

Our data indicated a potential MAC evasion mechanism in MAC-negative isolates. Bacteria inhibited the complement cascade and presented higher serum resistance. They were also more virulent, as they carried more virulence-related genes.

Paper III

Pan-drug-resistant *Klebsiella pneumoniae* isolated from Ukrainian war victims remain hypervirulent.

Background

Klebsiella pneumoniae is a human pathogen associated with high mortality and worrisome AMR as bacteria have acquired MDR, XDR, and even PDR. The bacterium

produces virulence factors to evade the complement-mediated lysis. Some reports showed that with increasing AMR, bacteria become more serum-sensitive.

In this study, we analyzed the serum resistance of 37 clinical AMR isolates obtained from patients in Ukraine.

Findings

A previous study showed that these isolates were resistant to many antibiotics, with nine isolates being colistin-resistant. Whole genome sequencing revealed resistance genes encoding the carbapenemases $bla_{\text{NDM-1}}$, $bla_{\text{NDM-1}}$ + $bla_{\text{OXA-48}}$, $bla_{\text{OXA-48}}$, $bla_{\text{NDM-6}}$, and $bla_{\text{NDM-1}}$ + $bla_{\text{KPC-3}}$. Genes associated with AMR towards other antibiotics were also found. Colistin-resistant bacteria carried the virulence genes *rmpA*, *rmpC*, *rmpD*, *iucA*, and *peg-344*, while the genes *ybt* and *iuc* were the most frequent in most isolates.

Colistin-resistant *K. pneumoniae* were significantly more virulent in the *Galleria mellonella* infection model at every tested concentration of bacteria. Similarly, bacteria with > 8 virulence genes killed more larvae than isolates with ≤ 8 genes. Moreover, colistin-resistant bacteria were more prone to infect mice in the pneumonia model. This indicated that resistant bacteria with more genes had an increased virulence.

Colistin-resistant isolates showed significantly better survival when tested for serum resistance than susceptible bacteria. Both colony counting and SYTOX Green signal measurements revealed higher serum resistance in colistin-resistant *K. pneumoniae*.

Our data demonstrated an association between colistin resistance, serum resistance, and overall virulence of *K. pneumoniae* towards the host. Worryingly, this correlation raises concerns about the treatment options for infections caused by hypervirulent bacteria. This indicates the need for further studies as the mechanism of colistin resistance presented in this paper is not fully understood.

Paper IV

Factor H-IgG chimeric proteins as a therapeutic approach against the gram-positive bacterial pathogen *Streptococcus pyogenes*.

Background

Streptococcus pyogenes, a gram-positive human pathogen, has developed several strategies to evade the complement system. Bacteria bind complement inhibitors C4BP and FH to suppress the activation and escape from the complement-mediated phagocytosis.

In this study, we evaluated the efficiency of fusion proteins as therapeutics against *S. pyogenes*.

Findings

S. pyogenes expresses proteins M and H, which bind human FH. Using wild-type and mutant strains, we observed that the FH6-7/hFc protein bound to bacteria via the expected FH domain, displacing serum FH and activating the complement system. The bacterium does not bind mouse IgG; therefore, we found that FH6-7/mFc binding to bacteria also occurred via FH domains. Proteins containing CCPs 18-20 showed only Fc-mediated binding and no functional effect. This confirmed that S. pyogenes mainly binds FH via CCPs 6-7.

Surface plasmon resonance data revealed strong nanomolar interaction between FH6-7/hFc and FH. Furthermore, we observed that proteins M and H bound similarly to FH and FH6-7/hFc. The interactions were primarily mediated by domains C and D in the bacterial protein H. Remarkably, the interaction between FH6-7/hFc and FH did not spontaneously activate complement in the fluid phase, nor did it affect hemolysis and platelet aggregation.

In the presence of FH6-7/hFc, bacteria were significantly better phagocytosed by immune cells. Additionally, FH6-7/hFc reduced bacterial growth in human blood by stimulating complement-mediated phagocytosis. The importance of complement in the killing was confirmed using complement and Fcγ receptor inhibitors. Finally, the administration of FH6-7/hFc significantly increased the survival rate of mice in the sepsis model.

Our data presented a successful method to target the evasion mechanism of *S. pyogenes*. Using fusion proteins, we could increase the complement activation and phagocytic

killing of the bacterium. That suggested the possibility of using fusion proteins against other bacteria, exploiting their virulence mechanisms against them.

Popular science summary

Man is constantly exposed to bacteria found in food, water, soil, our skin, and the gastrointestinal tract. While most bacterial infections are mild, some can lead to life-threatening diseases like pneumonia and bloodstream infections. In 2019, a global analysis estimated that 33 different bacteria were responsible for 13.7 million infection-related deaths, including 7.7 million direct deaths. That means bacterial infections accounted for one in eight (13.6%) deaths that year.

Our immune system, compromising innate and adaptive systems, protects us from these pathogens. Innate immunity includes physical barriers like skin and mucous membranes, preventing bacterial entry. When these barriers are breached, bacteria can invade the body and start the infection. Once microbes enter the body, immune cells and the complement system target the invaders. Specialized immune cells, called professional phagocytes, include monocytes, macrophages, and neutrophils. They detect specific molecules on the bacterial surface, engulf bacteria, and kill them in a process called phagocytosis. They can also produce particular molecules, called cytokines and chemokines, which induce inflammation, a hallmark of infection.

The complement system, a part of innate immunity, protects against bacteria and consists of plasma proteins that can be activated as a cascade. The primary functions of the complement system include labeling bacteria for phagocytosis, attracting phagocytes to the infection site, and directly killing some bacteria. Labeling for phagocytosis is a critical process, as it targets all types of bacteria.

However, bacteria develop mechanisms to avoid the complement. They try to hide from the system by hijacking complement proteins and producing proteins that block complement activation. Additionally, they degrade complement molecules to avoid being killed. These mechanisms, known as virulence factors, enhance bacterial virulence – the ability to infect and cause diseases in the host. Human pathogens *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes* have developed many strategies to escape from the complement. Therefore, we studied these bacteria, aiming to understand their mechanisms better.

Our study showed that *A. baumannii* produces a sugary capsule that physically protects bacteria from complement-mediated killing. Besides, some bacteria blocked complement activation to stay unharmed. Finally, by analyzing bacterial DNA, we identified potential virulence factors that we want to study in the future.

A *K. pneumoniae* study revealed alarming antibiotic resistance, including resistance to colistin, a last-resort treatment often used in hospitals. Worryingly, the colistin-resistant bacteria were also significantly more virulent, resisting complement-mediated killing and being more contagious in animal models.

We intended to overcome complement resistance using the specialized fusion proteins designed by our collaborators. These proteins proved effective against *S. pyogenes* by increasing complement activation and bacterial killing. When used as a therapeutic tool, the proteins protected mice infected with the pathogen.

Unfortunately, we are entering an era of antibiotic resistance, with an increasing number of bacteria becoming resistant to treatment. The World Health Organization recognizes this massive problem and asks researchers to propose solutions. We need to focus on creating treatment options that are not based solely on antibiotics.

Our findings highlight the need to continue researching evasion strategies in human pathogens. Although we identified many mechanisms, many more are waiting to be discovered and utilized against bacteria. Our fusion proteins use one of the known evasion strategies of *S. pyogenes* directly against the pathogen. Similar methods can be applied against other bacteria. I believe that our research could help develop novel treatment options.

Populärvetenskaplig sammanfattning

Människor utsätts ständigt för bakterier som finns i mat, vatten, jord, på huden och i mag-tarmkanalen. De flesta bakterieinfektioner är lindriga, men vissa kan leda till livshotande sjukdomar såsom lunginflammation och infektioner i blodomloppet. Under 2019 uppskattades det i en global analys att 33 olika bakterier var ansvariga för 13,7 miljoner infektionsrelaterade dödsfall, varav 7,7 miljoner direkta dödsfall. Det innebär att bakterieinfektioner stod för vart åttonde (13,6%) dödsfall det året.

Vårt immunförsvar, som består av medfödda och adaptiva system, skyddar oss mot dessa patogener. Det medfödda immunförsvaret innefattar fysiska barriärer som hud och slemhinnor, som förhindrar att bakterier tar sig in. När dessa barriärer bryts kan bakterier ta sig in i kroppen och starta en infektion. När mikroberna kommer in i kroppen angriper immuncellerna och komplementsystemet inkräktarna. Specialiserade immunceller, så kallade professionella fagocyter, inkluderar monocyter, makrofager och neutrofiler. De känner igen specifika molekyler på bakteriernas yta, slukar bakterierna och dödar dem i en process som kallas fagocytos. De kan också producera särskilda molekyler, så kallade cytokiner och kemokiner, som framkallar inflammation, vilket är ett kännetecken för infektion.

Komplementsystemet, som är en del av det medfödda immunförsvaret, skyddar mot bakterier och består av plasmaproteiner som kan aktiveras i en kaskad. Komplementsystemets primära funktioner är att märka bakterier för fagocytos, locka fagocyter till infektionsområdet och att direkt döda vissa bakterier. Märkning för fagocytos är en kritisk process för komplementet, eftersom den riktar sig mot alla typer av bakterier.

Bakterier utvecklar dock mekanismer för att undvika komplementet. De försöker gömma sig från systemet genom att kapa komplementproteiner och producera proteiner som hindrar komplementaktiveringen. Dessutom bryter de ned komplementmolekyler för att undvika att dödas. Dessa mekanismer, som kallas virulensfaktorer, ökar bakteriens virulens, det vill säga förmågan att infektera och orsaka sjukdomar hos värden. Bakterier som infekterar människor såsom *Acinetobacter*

baumannii, *Klebsiella pneumoniae* och *Streptococcus pyogenes* har utvecklat många strategier för att undkomma komplementet. Därför har vi studerat dessa bakterier för att bättre förstå deras mekanismer.

Vår studie visade att *A. baumannii* producerar en sockerkapsel som fysiskt skyddar bakterierna från komplementmedierad avdödning. Dessutom blockerade vissa bakterier komplementaktivering för att förbli oskadda. Slutligen, genom att analysera bakteriernas DNA identifierade vi potentiella virulensfaktorer som vi vill studera i framtiden.

En studie om *K. pneumoniae* avslöjade alarmerande antibiotikaresistens, inklusive resistens mot kolistin, vilket är en sista utväg som ofta används på sjukhus. Oroväckande nog var de kolistinresistenta bakterierna också betydligt mer virulenta och motstod komplementmedierad avdödning samt var mer smittsamma i djurmodeller.

Vi hade för avsikt att övervinna komplementresistensen med hjälp av de specialiserade fusionsproteiner som våra samarbetspartners tagit fram. Dessa proteiner visade sig vara effektiva mot *S. pyogenes* genom att öka komplementaktiveringen och bakteriedödandet. När proteinerna användes som läkemedel skyddade de möss som infekterats med bakterien.

Tyvärr är vi på väg in i en era av antibiotikaresistens, där allt fler bakteriearter blir motståndskraftiga mot behandling. Världshälsoorganisationen (WHO) är medveten om detta enorma problem och ber forskare att föreslå lösningar. Vi måste fokusera på att skapa behandlingsalternativ som inte enbart baseras på antibiotika.

Våra resultat understryker behovet av att fortsätta forska om undvikandestrategier hos patogener som drabbar människor. Även om vi har identifierat många mekanismer finns det många fler som väntar på att upptäckas och som kan utnyttjas mot bakterier. Våra fusionsproteiner använder en av de kända undvikandestrategierna hos *S. pyogenes* direkt mot patogenen. Liknande metoder kan tillämpas mot andra bakterier. Jag tror att vår forskning kan bidra till att utveckla nya behandlingsalternativ.

Populairwetenschappelijke samenvatting

Mensen worden voortdurend blootgesteld aan bacteriën in voedsel, water, aarde, onze huid en het maagdarmkanaal. Hoewel de meeste bacteriële infecties mild zijn, kunnen sommige leiden tot levensbedreigende ziekten zoals longontsteking en bloedbaaninfecties. Een wereldwijde analyse in 2019 schatte in dat 33 verschillende bacteriën verantwoordelijk waren voor 13,7 miljoen infectie-gerelateerde sterfgevallen, waaronder 7,7 miljoen directe sterfgevallen. Dit betekent dat bacteriële infecties dat jaar verantwoordelijk waren voor één op de acht (13,6%) sterfgevallen.

Ons immuunsysteem, dat bestaat uit aangeboren en verworven systemen, beschermt ons tegen bacteriën. Aangeboren immuniteit bestaat uit fysieke barrières zoals huid en slijmvliezen die bacteriële binnendringing voorkomen. Wanneer zulke barrières worden doorbroken, kunnen bacteriën het lichaam binnendringen en kan er een infectie ontstaan. Wanneer bacteriën het lichaam binnendringen, richten onder andere immuuncellen en het complementsysteem zich op deze indringers. Professionele fagocyten (o.a. monocyten, macrofagen en neutrofielen) zijn gespecialiseerde immuuncellen die specifieke moleculen op het bacteriële oppervlak detecteren. Deze fagocyten omsluiten bacteriën en doden ze in een proces dat fagocytose wordt genoemd, ze produceren ook moleculen als cytokinen en chemokinen die ontsteking veroorzaken, een kenmerk van infectie.

Het complementsysteem, een onderdeel van het aangeboren immuunsysteem, beschermt tegen bacteriën en bestaat uit plasma-eiwitten die als een cascade geactiveerd kunnen worden. De belangrijkste functies van complement zijn het labelen van bacteriën voor fagocytose, het aantrekken van fagocyten naar de infectieplaats en het direct doden van bepaalde bacteriën. Het labelen van bacteriën voor fagocytose is een cruciaal proces omdat het geen onderscheid maakt tussen soorten bacteriën.

Bacteriën hebben echter mechanismen ontwikkeld om het complement systeem te omzeilen, ze proberen zich voor het systeem te verbergen door complementeiwitten te kapen of door eiwitten te produceren die activatie van het complement systeem blokkeren. Een andere manier die bacteriën gebruiken om te voorkomen gedood te worden door het complement systeem is het afbreken van complementmoleculen. Deze mechanismen, bekend als virulentiefactoren, vergroten de virulentie van bacteriën - het vermogen om de gastheer te infecteren en ziektes te veroorzaken. Humane ziekteverwekkers als *Acinetobacter baumannii*, *Klebsiella pneumoniae* en *Streptococcus pyogenes* hebben veel strategieën ontwikkeld om aan het complement systeem te ontsnappen, daarom hebben we deze bacteriën bestudeerd om de mechanismen die zij hebben om het complement systeem te omzeilen beter te begrijpen.

Ons onderzoek toonde aan dat *A. baumannii* een suikerhoudende capsule produceert die bacteriën fysiek beschermt tegen complement-gemedieerde doding. Daarnaast hebben we aangetoond dat sommige A. baumannii stammen complement activatie blokkeerde om te overleven. Tot slot identificeerden we door het analyseren van bacterieel DNA potentiële virulentiefactoren die we in de toekomst willen bestuderen.

Een onderzoek naar *K. pneumoniae* onthulde alarmerende antibioticaresistentie, waaronder resistentie tegen colistine, een laatste redmiddel dat vaak in ziekenhuizen wordt gebruikt. Verontrustend genoeg toont ons onderzoek aan dat colistine-resistente bacteriën ook significant virulenter waren, deze bacteriën weerstonden complement-gemedieerde doding en waren besmettelijker in diermodellen.

Ons plan was om complement-resistentie te bestrijden met behulp van gespecialiseerde fusie-eiwitten ontworpen in een laboratorium waar wij een samenwerkingsverband mee hebben. Deze eiwitten bleken effectief tegen *S. pyogenes* door verhoging van complement-activatie en vervolgens het doden van de bacterie. Wanneer deze eiwitten werden gebruikt als therapie in *S. pyogenes*-geïnfecteerde muizen identificeerde wij dat zij een beschermend effect hadden.

Helaas is antibioticaresistentie een groeiend probleem, en worden steeds meer bacteriën resistent tegen antibiotica. De Wereldgezondheidsorganisatie erkent dit enorme probleem en vraagt onderzoekers om met nieuwe oplossingen te komen. We moeten ons richten op het creëren van behandelingsopties die niet alleen gebaseerd zijn op antibiotica.

Onze waarnemingen benadrukken de noodzaak om door te gaan met onderzoek naar immuun-ontwijkingsstrategieën van menselijke ziekteverwekkers. Hoewel we veel van

zulke mechanismen hebben geïdentificeerd, is de verwachting dat er nog vele onontdekt zijn en tegen bacteriën gebruikt kunnen worden. Onze fusie-eiwitten gebruiken bijvoorbeeld één van de bekende ontwijkingsstrategieën van *S. pyogenes* rechtstreeks tegen de bacterie, vergelijkbare methoden zouden kunnen worden toegepast tegen andere bacteriën. Ik geloof dat ons onderzoek kan helpen bij het ontwikkelen van nieuwe behandelingsmogelijkheden.

General discussion and future perspectives

Treatment options

The main idea of this project was to elucidate the mechanism of complement system evasion in human pathogens in order to develop treatments against them.

We have tested the FH fusion protein against a well-known mechanism in *S. pyogenes*. The protein successfully displaced serum-acquired FH from the bacterial surface and mediated complement opsonization. As mentioned in the previous chapter, proteins comprised of FH and C4BP domains have also been proven efficient against other bacteria, such as *Neisseria* or *Haemophilus*. Naturally, we wanted to employ proteins against *A. baumannii* since one paper claimed that *A. baumannii* uses OmpA to bind FH to evade complement [174]. Despite repeated experiments, we could not detect any significant binding of FH or C4BP compared to *S. pyogenes* or *M. catarrhalis* used as controls. Therefore, our efforts to use fusion proteins containing FH or C4BP domains would be in vain, and other approaches must be considered.

Could fusion proteins be used against other pathogens known to bind complement inhibitors? One example might be a gram-positive *Staphylococcus aureus*, which acquires serum FH using surface protein SdrE. The FH18-20/hFc fusion protein used against this pathogen displayed similar complement activation properties as against other bacteria [279]. There are also many FH-binding gram-negative pathogens [147]. Therefore, displacement of inhibitors could not only opsonize them for phagocytosis but could also lead to MAC-mediated lysis. We may consider testing this against other bacteria, such as *Pseudomonas aeruginosa* or *Yersinia enterocolitica*. Could we also use fusion proteins against *Klebsiella*? The literature does not describe the binding of FH or C4BP. However, one study reported the incidence of sialylation in the bacterial capsules of hypervirulent *Klebsiella [217]*. Sialic acid on the surface of *Neisseria* mediates FH binding; could it then mediate binding in *Klebsiella*?

So far, we have considered fusion proteins comprised of FH or C4BP domains. Can we alter the domain fused to the antibody? Vitronectin is also known to be acquired by pathogens to inhibit MAC formation. We can speculate that Vn/hFc could displace the serum-acquired inhibitor from the surface of *Haemophilus* or *Pseudomonas* [280]. What about other proteins such as fibrinogen, plasminogen, or EMC proteins [280-282]? Would it be possible to generate decoy fusion proteins, which could bind virulence factors expressed on pathogens? They could capture surface proteins on bacteria, blocking their ability to bind actual host proteins. At the same time, their exposed Fc domains could activate the complement and Fc receptors. However, it would be necessary to identify the exact binding sites that pathogens recognize. Theoretically, this approach could work against different virulence factors, as bacteria produce several binding proteins.

Of course, the identification of evasion mechanisms would help develop treatment options other than fusion proteins. Many ongoing trials are evaluating monoclonal antibodies against various pathogens, including ESKAPE bacteria [283]. Choosing a suitable target to raise the antibodies against is crucial. Bacteria produce many antigens, but it is worth remembering that not all antigens are expressed equally. Some factors are expressed at different stages of infection, as they are used for different mechanisms. Therefore, one option would be to target at least two antigens, which can be achieved using bispecific antibodies such as those against *Pseudomonas* virulence factors PcrV and Psl [284]. Recognition of two antigens would increase the efficiency of the antibody since targeting a single antigen has often been shown insufficient to provide adequate protection. Remarkably, one group developed a multivalent fusion antibody targeting five *S. aureus* surface proteins [285]. In addition to binding to surface proteins, the fusion antibody resisted the proteolysis by GluV8, avoided binding by SpA and Sbi, and neutralized pore-forming leukocidins expressed by the pathogen. While doing so, the antibody maintained the complement and Fc receptor activation functions. Moreover, the antibody cooperated with vancomycin, enhancing the clearance of *S. aureus*. It is astonishing how many functions this antibody had compared to a standard single-antigen antibody.

Finally, vaccines would be a powerful tool against bacterial infections. Vaccines would be valuable in preventive medicine to protect against pathogens such as *S. pyogenes*, which infect and cause disease also in healthy individuals. It is shocking that despite decades of research, no effective vaccine against GAS has been developed. However, the increase in AMR also demonstrates the requisite for vaccination against other bacteria.

Pathogens such as *A. baumannii* and *P. aeruginosa* are at the top of the priority list for new treatment options. However, they are far behind in the vaccine development pipeline. A study from 2023 calculated a total of 94 vaccine preclinical vaccine candidates and 61 development candidates, but these pathogens were only tested for a couple of preclinical vaccine candidates [286]. Unfortunately, the most dangerous bacteria are the most challenging to develop new vaccines against. They usually target immunocompromised patients; thus, they are associated with high mortality in a relatively small population, making any potential clinical trial more problematic. Additionally, the efficacy of vaccines tested so far has not been underwhelming, regardless of the approach and immunogen tested.

A new potential approach could be to use the mRNA vaccine platform to target the most problematic bacteria [287]. This system proved successful during the recent COVID-19 pandemic. The mRNA vaccines have been tested against a few pathogens to combat bacterial infections. An mRNA vaccine encoding heat-shock protein 65 reduced the bacterial load of *Mycobacterium tuberculosis* in a mouse model. Similarly, a vaccine based on the F1 capsule antigen of *Y. pestis* protected mice from the infection after only a single dose. Generally, mRNA vaccines induce antibody production and activation of $CD4^+$ and $CD8^+$ T cells. However, they mediate the response to only one protein antigen, which may be insufficient to target more complex virulence factors. Additionally, bacterial biodiversity and evasion mechanisms may complicate vaccine efficacy [287].

Taken together, these studies show promising results. It is reassuring that the problem of bacterial infections and AMR is being recognized and that the research community is trying to provide answers. Nevertheless, we must continue investigating human pathogens, deciphering their evasion mechanisms, and using them against bacteria.

Future perspectives

In the near future, we want to continue studying *A. baumannii* complement evasion since it is a relatively new bacterium in the complement field with little interest compared to *S. pyogenes* and *K. pneumoniae*. It produces a variety of virulence factors, but not many have been confirmed to support complement evasion.

Our results indicated the presence of an evasion strategy as we observed inhibition of AP and, consequently, no MAC deposited on several isolates. We could not pinpoint the inhibited step, as the C3 and C5 convertases are structurally similar. We speculated that the capsule may interfere with AP activation. Possibly, the capsule disturbed the

C3 convertase and C3b deposition; therefore, the C5 convertase could not be formed, and C5 was not cleaved to initiate MAC formation. We want to generate capsule mutants out of clinical isolates to prove this. Unfortunately, despite our best efforts, we could not mutate clinical isolates of *A. baumannii*. To our surprise, bacteria did not acquire plasmids used for transformation. That was indeed surprising, as the bacterium is known to spread virulence factors using horizontal gene transfer. Interestingly, our collaborators and colleagues we have met at conferences confirmed having similar problems with transforming *A. baumannii*. It might be that the solution is using a conjugation mechanism to transform bacteria. Perhaps the clinical isolates are more prone to receive plasmids in this manner than standard electroporation.

Our other approach to tackle complement system evasion will be to express *Acinetobacter* proteins involved in serum resistance. Some virulence factors have been characterized as affecting the serum survival of the pathogen, as the mutant strains were more susceptible to complement. We want to produce proteins, such as CpaA, PAL, SurA1, PLC, and PLD, to test them in the complement activation assays. The enzyme CpaA has already been identified to cleave CD46 and CD55, so we speculate it may also degrade other complement molecules. Once we identify the mechanism, we will express the protein in another gram-negative bacterium sensitive to human serum to analyze potential acquired resistance.

Moreover, we plan to investigate OMVs produced by *A. baumannii*. The bacterium is known to secrete vesicles containing virulence factors, such as OMPs, LPS, and other currently unidentified peptides. OMVs are also used to spread AMR. We speculate that OMVs may play a role in complement resistance by carrying virulence factors affecting the activation or acting as a decoy for complement deposition, as they carry antigens recognizable by the complement system.

Finally, we observed that *A. calcoaceticus* isolates bound human C3. This binding was characterized only for this strain, leaving us to speculate on the reason for this interaction. C3 binding has been identified as an evasion mechanism in other pathogens, such as *Moraxella catarrhalis* and *Staphylococcus pyogenes*, but we do not know its function in *A. calcoaceticus*. This strain is not considered as pathogenic as *A. baumannii*; therefore, C3 binding may support its persistence and colonization of the host.

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