The roles of the anaphylatoxin receptors during invasive disease as well as mucosal colonization caused by *Neisseria meningitidis*

Die Rolle der Anaphylatoxinrezeptoren während invasiver Infektion sowie mukosaler Kolonisation verursacht durch *Neisseria meningitidis*

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1. Introduction

1.1. Invasive Meningococcal Diseases

Invasive Meningococcal diseases (IMD), caused by the human specific pathogen *Neisseria meningitidis* (*Nme*, meningococci), can manifest as meningitis, septicemia or both (1). The first documented deaths by meningococcal diseases might have been described as early as the 17th century (2). R. Bruce Low suggested 1899, that back then, the disease might have often been documented under different names, for example one classification could have been called "spotted fever and purples" (2) and might have in fact described deaths from meningococcal diseases (2). The very first reports of epidemic meningococcal infections, at that time called cerebrospinal fever, date as far back as the year 1805 in Geneva, documented by M. Vieusseux (3) and 1806 in Massachusetts, New England (2). R. Bruce Low described both as the first meningococcal disease outbreak studies that rose awareness in the medical society and lead to many more documented outbreaks from that time onwards (2). Later in the 19th century a discussion arose, whether the disease can be transmitted from human to human or not (2) and it should take another 82 years, after the first epidemic publication by Vieusseux, for meningococci to be discovered. In 1887 Anton Weichselbaum was able to characterize meningococci, at that time called "diplococcus intracellularis", as the causative agent of meningococcal disease cases that he studied (4). His observations were afterwards proven by other scientists, including H. Jaeger (5), who together with Dr. Scherer also postulated, that the transmission of *Nme* occurs via nasal discharges (5). Even though meningococcal diseases are known since over 200 years and *Nme* were discovered over 100 years ago, nowadays people still die from meningococcal diseases and a lot about the bacteria and the infections is still unknown. Especially, as new links between meningococcal infections and for example anaphylatoxins, released by the complement system, are being established, further studies as this thesis are still very important in the 21th century to shed further light onto the multitude of interactions between these commensal- or causative agents and the hosts immune system.

1.1.1. *Neisseria meningitidis* in General

The causative pathogen, *Neisseria meningitidis*, were isolated for the first time in 1887 by Anton Weichselbaum (4, 6). The diplococcus *Nme* are capsulated gram-negative bacteria, which are classified into serogroups depending on the expression of different capsular polysaccharides (6). The necessary genes for the capsule formation are located for all serogroups in the *cps* locus. The different regions of this locus are similarly arranged and the region A holds the genes, which determine the expression of the different capsular polysaccharides. Thereby the *Nme* are classified in 12 different serogroups: A, B, C, E, H, I, K, L, W, X, Y and Z. Originally, a thirteenths serogroup "D" was described, however genome analysis by Harrison and colleagues revealed that it is an unencapsulated serogroup C (7). Out of these 12 serogroups, only 6 (A, B, C, W, X and Y) are associated with IMD. The capsule is the most important virulence factors of *Nme* as most strains associated with IMD are encapsulated (7). *Nme* express a variety of further virulence factors, that are important for different functions. The endotoxins expressed by *Nme* are lipooligosaccharides (LOS), which are important in the interaction with the host immune system (8). For adhesion *Nme* express pili (9), Opa (10), Opc (11), neisserial adhesin A (NadA) (12), *Neisseria* hia homologue A (NhhA) (13), adhesion and penetration protein (App) (14) or meningococcal serine protease A (MspA) (15). For iron sequestration *Nme* express the hemoglobin receptor (HmbR) (16), the hemoglobin-haptoglobin receptor (HpuAB) (17), the transferrin receptor complex comprised of transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB) (18), the lactoferrin receptor complex composed of the two lactoferrin-binding proteins (LbpA and LbpB) (19, 20) as well as porins (PorA, PorB) for uptake of further nutritions (21). Additionally, *Nme* have evolved special mechanisms as for example phase variation, to quickly change antigen structures (22), horizontal gene exchange mechanisms, to acquire further virulence factors (23), and molecular mimicry, to mask for example polysialic acid (PSA) in serogroup B *Nme*, which has a similar structure to PSA found in neural cell adhesions molecules (24, 25).

1.1.2. *Neisseria meningitidis* as a Commensal Bacterium and a Causative Agent

Meningococci are human specific bacteria and are found as commensals in 8-25% of the world's population. They are transmitted via droplets or salvia and their normal habitat is the human upper respiratory tract. *Nme* express a great variety of adhesins, that bind to different host receptors, allowing them to colonize the mucosal tissue of the human upper respiratory tract. Meningococcal carriage is mostly asymptomatic. How they pass the mucosal barrier and what triggers them to become invasive, is still a topic of debate, with extensive research being

conducted upon (26). IMD can occur very quickly after transmission and can already be lethal after first symptoms within 12- 24 hours in case of septicaemia (27). Once *Nme* have entered the blood stream they quickly increase in numbers, cause sepsis or even cross the blood-meningeal barrier and cause meningitis (26). The current situation of IMD in Germany is regularly published by the Robert Koch-Institut. In 46% of the reported IMD cases, patients developed a meningitis with clinical symptoms of neck stiffness, increased intracranial pressure and *Nme* detection in cerebrospinal fluid. A meningitis together with a sepsis manifested in 14% of the patients and a sole sepsis in 40% of the patients. Clinical symptoms of meningococcal sepsis are petechiae and *Nme* detection in the patient's blood. (28) IMD, either manifested as meningitis or septicemia, has to be treated as early as possible with antibiotics such as penicillin, cefotaxime or ceftriaxone administered parenterally for 5-7 days (29). Even with these available treatments the overall mortality rate of IMD is still as high as 10%. The mortality rate of meningitis alone is between 1-5% and in contrast to that the mortality rate of fulminant sepsis varies between 20 to 80%, depending on the study (27). According to the Robert-Koch-Institut, in Germany the mortality rate of IMD is still at 10%, even with the available treatments in intensive care units. For meningococcal meningitis the mortality rate is at 2,1% and for sepsis at 18%. In patients that develop a Waterhouse-Friderichsen syndrome (WFS, see below) the mortality rate is as high as 38% (28). One characteristic symptom of fulminant sepsis are hemorrhagic skin rashes and purpura fulminans (PF) (27). Meningococcal LOS can function as a strong activator of the coagulation pathways resulting into PF (30). Due to the strong activation of the coagulation cascades, the factors present are used up leading to strong bleeding, which can cause of necrosis and may make it necessary to amputate whole limbs. A particular severe complication during IMD is the WFS. When *Nme*-induced vascular damage occurs in the adrenal glands, the resulting tissue injury can lead to a reduced amount of cortisone release in the patient (27). In WFS the lethality is therefore severely increased to 38% (28). Even among the survivors of IMD, up to 19% will suffer from sequelae such as neurological damage, chronic pain, skin scarring, seizures, motor defects, visual impairment, hearing impairment, conjunctivitis, septic arthritis, hearing loss or amputations (29).

1.1.3. *Neisseria meningitidis* Epidemiology

Transmission of *Nme* occurs via droplets or direct contact. IMD can be severe, however most people will carry *Nme* asymptomatically as a commensal bacterium in their nasopharynx. The overall carriage rate of *Nme* is around 8-25% (26), but can strongly differ among different ages and groups (27). In infants a carriage rate of 4,5% was observed, increasing with age to a peak

of 23,7% in young adults at around 19 years of age (31). An increased carriage rate can also be observed in groups of people with close contact, such as in student dormitories (32) or military camps (33). *Nme* carriage is not directly linked to IMD, as only a small portion of meningococcal strains are associated with IMD. The incidence of IMD also greatly depends on age and region. In Europe for example, the incidence rate is the highest in infants being younger than 1-year-old with 8,2 cases per 100.000. The incidence rate between 1 and 4 year old toddlers is at 2,5 cases per 100.000 and another peak can be observed among 15-24 year old's, according to the "European Centre for Disease prevention and control" (34). Among all ages, the worldwide incidence rate of IMD is below 5 in 100.000 inhabitants (35). The European Economic Area has an incidence rate of around 0,6 cases per 100.000 inhabitants (34), with Germany having around 0,4 cases per 100.000 inhabitants (28) and the United Kingdom having the second highest incidence rate with the Netherlands of 1,2 cases per 100.000 inhabitants in Europe (34). In the United States the incidence rate is at 0,53 per 1000.000 inhabitants (36). Worldwide the highest incidence rates, of more than 10 cases per 100.000 inhabitants, can be found in Africa. During outbreaks, these rates even increase to 100-1.000 cases per 100.000 inhabitants. These severe outbreaks periodically occurred in the past in an area between Senegal and Ethiopia. This region was therefore termed "meningitis belt". In contrast to other outbreaks around the globe, which often occur during cold seasons, in Africa outbreaks occur during dry season (35). But not only the incidences are different across the world, also the strains causing these infections are different depending on the region. Whereas the serogroups B and C are predominantly responsible for IMD outbreaks, in the USA also serogroup Y is more often found. In Asia, IMD is most often caused by serogroups A and C. In the meningitis belt area in Africa on the other hand, A, W, C and X are most often found to cause IMD (37). These regional differences are especially important for strategies to fight IMD globally. In 2010 MenAfriVac, a monovalent group A meningococcal conjugate vaccine, was introduced to the meningitis belt. Before that, almost all IMD outbreaks in that area were caused by serogroup A meningococci. The biggest outbreak documented, occurred in 1996 and 1997 in the meningitis belt, when 250.000 people were infected and 50.000 died from IMD. The introduction of MenAfriVac drastically reduced the amounts of IMD caused by serogroup A meningococci in the meningitis belt (35). Similar achievements could be observed with the introduction of vaccinations against serogroup C meningococci in Europe (38). In addition, the introduction of the newly developed vaccine against serogroup B meningococci from 2013, could be helping to further decrease IMD cases (34, 39).

1.2. Meningococci and the Immune System

Multicellular organisms have developed several strategies for defense against infectious agents such as meningococci. All those mechanisms are normally allocated to either the innate immunity, if they are protecting against a greater variety or to the adaptive immunity, if they are targeting pathogens in a highly specific and adaptive manner. Those mechanisms range from rather unspecific epithelial and chemical barriers, cellular components like phagocytic cells or the complement system, allocated to the innate immunity, to more individually directed cellular processes being started by the production of cytokines and specific antibodies (40).

1.2.1. The Complement System

One important part of the rather wide targeting innate immunity is the complement system. Its successfulness has been proven by the fact, that genetic analysis traced back the evolution of the mammalian complement system to over 500 million years ago and single components even further (41). Discovered was this ancient system over a century ago by Jules Bordet. He observed that serum must contain heat labile components, capable of mediating bacterial lysis and was awarded for his achievements in 1919 with the Nobel Prize in Physiology or Medicine (40, 42). A lot of research since then has discovered that the complement system, preliminary thought to be only involved in bacterial lysis, is actually entangled in many important processes (43). For example, the complement receptors 1 (CD35) binds C3b, is expressed on erythrocytes and allows thereby the transport of immune complexes, while CD35 expression on phagocytic cells triggers phagocytosis (44). The complement receptor 2 (CD21) on the other hand is expressed as a co-receptor on B cells, enhancing B cell receptor signaling, and on follicular dendritic cells for the development of memory B cells. Meaning that the complement system via CD21 even establishes a direct link between the innate and the adaptive immunity (42, 44). Another important role of the complement system is the maintenance of homeostasis versus eliciting a full immune reaction upon danger sensing (43). The last example of the complement function is of upmost importance and is explained in more details below, as it’s the core element of this work.

1.2.2. Complement activation and MAC formation

The complement system consists of more or less one common cascade leading to the final assembly of the MAC. This cascade can be activated via three different pathways, named the alternative-, the classical-, or the lectin pathway. The alternative pathway is started by a constant tick-over process in which a thioester bond of C3 is spontaneously hydrolyzed forming

$C3(H_2O)$. Next, this bioactive $C3(H_2O)$ binds to factor B. This complex is then cleaved by factor D, forming the fluidic phase C3-convertase $C3(H_2O)Bb$, which cleaves C3 into C3b and C3a. The classical pathway is mediated via antibody antigen binding by IgM or IgG and subsequent binding of the C1 complex (45). The polymeric IgM is only exposing Fc binding sites upon antigen binding and the monomeric IgG is binding only strongly enough to the C1 complex in a hexameric conformational setting, thereby ensuring that the classical pathway is only activated upon antibody-antigen binding (46). The C1 complex is comprised of C1q, C1r and C1s. Fc binding is done by C1q, resulting in conformational changes of the whole complex, leading to autoactivation of C1r. C1r then activates C1s and the catalytic domain of C1s cleaves C4 into C4a and C4b and C2 into C2a and C2b. The third mechanism, the lectin pathway is activated via binding of different pattern recognition molecules, the mannose-binding lectin (MBL) or ficolins, to structures normally found on apoptotic cells, viruses or bacteria. MBL and ficolins are associated with MBL-associated serine proteases, which are activated in a process similar to the C1 complex and also cleave C4 into C4a and C4b and C2 into C2a and C2b. All three pathways lead upon activation to the assembly of a C3-convertase for subsequent C3 cleavage. In the alternative pathway, the C3-convertase consists of C3b binding to Factor B, which is cleaved via activated Factor D into Bb and Ba leaving a complex of C3bBb on the membrane surface. The same mechanism is involved in the formation of the C3-convertases in fluidic phase during the tick-over process. The primary C3-convertase from the classical and lectin pathway on the other hand, consists of the cleavage product C4b, covalently binding to the membrane surface and the attached C2a fragment. These primary C3-convertases then cleave C3 into C3a and C3b. While C3a is released, C3b further opsonizes the detected surface or is incorporated into the C3-convertases itself for forming the C5-convertase. The C5-convertase is responsible for cleavage of C5 into C5a and C5b. C5a is released and C5b binds to the complement components C6, C7 and C8, linking the complex to the surface and ultimately leading to the recruitment of C9 molecules. Multiple C9 molecules are then finally inserted into the membrane, forming the so-called MAC, creating a hole of around 10 nm in the targeted cell wall. The targeted cell is then being lysed via these MACs (45). This final MAC formation is of utmost importance in fighting off *Nme*. In fact, the risk of getting IMD is increased by a factor of 10.000 in patients with a deficient C7 complement factor (47).

1.2.3. Complement mediated antigen detection by immune cells

In the context of being a sensory system, the complement system is involved in starting immune processes, providing continuous stimuli for prolonged reactions and marking of pathogens.

Phagocytosis of foreign particles is an important part of the innate immune system and is initiated via pattern recognition receptors (PRRs) or complement mediated opsonization of these particles (40). Through the tick-over process and C3-convertases, C3b is continuously generated and can then be incorporated into C3-convertases or is further cleaved into iC3b, which does not have this capability anymore. C3b and C4b, produced in the classical- or lectin pathway, can then covalently bind to foreign surfaces. These molecules can then be recognized via their corresponding C3b-receptor, labeled as CD11b/CD18 and the C4b-receptor, better known as CD11c/CD18. Both receptors are highly expressed on monocytes/macrophages and neutrophils (45). These phagocytic cells are then able to uptake the opsonized particles, for example bacteria, for degradation and subsequent antigen display in case of monocytes/macrophages and dendritic cells. This results in the efficient clearing of the infection or also in a full elicited immune reaction with antigen presentation and long-term immunization (40). With this indirect route of bacterial killing, its prominent roles in inflammation, the adaptive immunity and the MAC formation, it becomes clear that the complement system has a pivotal role in many different pathological conditions and also the homeostasis (40).

1.2.4. Complement in health and disease

The complement system has multiple functions and one of the most central ones is the constant surveillance of the organism for foreign elements. As mentioned above, C3b is continuously produced in a tick-over process, leading to opsonization of foreign surfaces or dying cells that are not marked as “self”. Dying cells can then be cleared by phagocytic cells. Foreign element detection on the other hand starts the different complement cascades, leading to the direct killing of pathogens by lysis and subsequent eliciting of inflammation processes for clearance. This process marks the second important role of the complement system, which is to trigger a full immune response via innate and adaptive immune reactions. As the complement is involved in such a brought array of immune functions, the complement’s contribution can have quite adverse outcomes in different pathological conditions (43). In wound healing for example, the pro-inflammatory condition of the complement activation can hinder the healing process, as an accelerated healing was observed in mice lacking C3 or C5aR1. Stavros Rafail and colleagues suggested from their murine experiments, that loss of C3 results in an increased beneficial accumulation of mast cells in the damaged tissue and that the lack of C5aR1 impaired the recruitment of further immunological cells. Both conditions resulted then in the observed accelerated wound healing (48). Such pro-inflammatory conditions are normally beneficial in clearing of bacterial infections, but once cleared or in other conditions, the inflammatory

responses also have to be stopped. Therefore, the complement system is likewise associated with immunosuppressive immune reactions. For example, it has been shown that signaling through the coreceptor CD46 with IL-2 receptor, mediated the transition of $CD4^+$ T cells to T-regulatory cells, which are capable of secreting the anti-inflammatory cytokine IL-10 (49). Another condition in which the complement is involved in is sepsis. In 2015 on the "Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3)" sepsis was defined as a severe organ dysfunction as a result of an uncontrolled reaction of the host to an infection (50). There are different underlying conditions that contribute to sepsis. Inflammatory responses are started upon detection of pathogen-associated molecular patterns (PAMPs) via PRRs or the complement system. Several immune responses are triggered leading to an increased release of different cytokines, chemokines and anaphylatoxins. In addition to pro-inflammatory reactions, also anti-inflammatory processes are started in sepsis and even a dysfunction of the endothelial barrier can be seen. Altogether, these different circumstances then result in the life-threatening sepsis (51).

1.2.5. Regulation of the complement system

As described above, the complement system mediates or elicits many different immune responses. Therefore, a tight regulation of the complement system is needed to ensure that its full capacity is only released if necessary. This regulation consists on the one hand on specific mechanisms to start the complement pathways and on the other hand on the expression of proteins on host cell membranes, to mark them as self and protect them from complement attacks. Activation of the classical- and lectin pathway depends on pattern recognition molecules, which are normally specific for foreign substances or danger molecules (45). For example, IgG or IgM dependent on specific antibody-antigen binding for C1q activation, which is needed for complement activation in the classical pathway. C1q can also bind many other charged patterns, however like for example C-reactive protein, some of these are suspected to have more important roles for example in clearance of apoptotic cells via phagocytosis (52). The lectin pathway is also depending on lectins binding to specific pathogenic patterns, like microbial polysaccharides. The alternative pathway contrary to that, is non-specifically initiated and then amplified via binding of C3b to hydroxyl- or amino groups, which can be found on pathogenic, as well as on mammalian cells (40). Therefore, a permanent protection of self-cells against the C3b deposition and subsequent complement activation must be ensured. Several soluble and membrane bound complement inhibitors take on this task. The most important player is factor H, which is a soluble inhibitor of the C3-convertase formation, and together

with other factors expressed on the cell membrane, these factors lead to the rapid inactivation of C3b into iC3b with the plasma serine protease factor I (53).

1.2.6. Immune evasion mechanisms of *Nme*

As the complement system is an important immune mechanism, pathogens have evolved an array of immune evasion strategies, from newly developed mechanisms to processes exploiting the cellular protection mechanism, that should normally protect host cells (54). For *Nme*, the most important virulence factor undoubtably is its capsule, as IMD is caused by 6 different encapsulated serogroups (7). There are several different strategies found in *Nme* to evade the complement system. Lectin pathway activation is minimized by a reduced binding of MBL on most *Nme* serogroups (55). The classical pathway activation is started by antibody-antigen binding, but can also be started via C1q binding directly to LPS for example. However, the LOS expressed on *Nme* shows a reduced binding towards C1q, also disabling a direct activation, as it can be observed in other gram-negative strains (56) Other important molecules for *Nme*, in counteracting the complement system, are factor H binding protein (57) and *Neisseria* surface protein A (NspA), which are both able to bind factor H, to protect themselves from the complement system (58), the protease NalP, which cleaves C3 atypical to its normal cleavage (59) or Porin A, which binds C4b-binding protein, which prevents the assembly of the C3-convertase in the classical pathway (60). To avert specific immune reactions mediated by antibodies, *Nme* apply antigenic and phase variation, allowing them to quickly switch genes off or on, in order to avoid being recognized by these structures (54).

1.2.7. Mast cells

Next to the direct killing of bacteria, the complement system as a danger sensing system is also closely entangled with different cell types such as phagocytic neutrophils (1.2.9) and monocytes/macrophages (1.2.8) or mast cells, which are capable of eliciting several different immune reactions (40). Mast cells are bone marrow derived cells that are in their mature state tissue residing. They are most commonly found in tissues that are protecting the human body from the environment, such as the skin or mucosal sites. They are characteristically filled with granules full of proteases and further mediators, which are quickly released upon mast cell activation. One of these mediators is histamine, which is directly soluble after release (61). Histamine activates vascular permeability, vasodilation and affects the heart rate. Due to these effects together with further molecules, mast cells play an important role in anaphylaxis. This quickly progressing immediate hypersensitivity reaction, often in response to certain food or venoms, can become a very serious condition and can even lead to death (40, 62). Because of

these devasting effects, that mast cells have in anaphylaxis and also in chronic diseases such as asthma, they are mostly known for their negative functions in these settings. However, nowadays it is established that mast cells also have several beneficial traits and are important for the immune system, as being very quick first responders. They are able to release their granules within seconds or minutes upon stimulation and can be activated by several different stimuli (61). They express IgE and IgG binding Fc-receptors on their cell surface, which allows them to react to the specific antigens targeted by these antibodies (63). Mast cells also express different TLRs, which belong to the PRRs, and are thereby able to directly react to pathogens. Additionally, they can also be activated by the anaphylatoxin C5a via the C5aR. By these different routes, mast cells are activated, release their granular contents within seconds to hours and can continue to produce several cytokines such as tumor necrosis factor (TNF)-a or IL-6, which are important for example for attraction of further immune cells like neutrophils (61). By their activation via C5a and strong chemoattraction to C3a and C5a, mast cell are also involved in processes directly linked to the complement system (64).

1.2.8. Monocytes and macrophages

Previously, it was thought that circulating monocytes, derived from hematopoietic stem cells, are precursors of macrophages and that their main function is the migration into tissue sites in order to develop into macrophages. Therefore, monocytes were mostly seen as a replenishing pool for macrophages in the periphery (40). Nowadays, it has been established that the relationship between monocytes and macrophages is more complex than thought of before (65). The major finding was that there are tissue resident macrophages, which grow during embryogenesis and are capable of replenishing the pool of tissue-resident macrophages by cell division without monocytes. Nevertheless, there are monocytes, that migrate into tissue sites and differentiate into monocyte-derived macrophages as previously believed. Additionally, there are also monocytes that home into tissue sites without differentiation into macrophages and act on their own (65). Monocytes and macrophages have versatile functions. They are both capable of phagocytosis. Clearing of apoptotic cells for maintaining homeostasis is mainly associated with macrophages. Phagocytosis of pathogens during infections is done by monocytes and macrophages for clearance as well as subsequent antigen presentation. They can present up taken antigens in an MHC-II dependent manner and therefore are called antigen presenting cells (APCs). This antigen presentation together with co-stimulation then enables them to start further adaptive immune process such as the activation of T-cells (65). Circulating monocytes have a half-life of around 20 h and macrophages of up to 3 weeks. Compared to

phagocytic neutrophils, which have a half-life span of around 11 h, macrophages are long living cells, which highlights their importance in maintaining homeostasis (66, 67). During inflammation circulating monocytes migrate into sites of infection. Upon danger signaling and antigen detection, monocytes and macrophages release several important cytokines (40). Macrophages release TNF, interleukin (IL)-6, IL-8, IL-12, IL-23 and type I interferons (68). Monocytes are known for releasing proinflammatory cytokines like TNF-α, IL-1β, IL-6, MIP-1β and IL-8 as well as anti-inflammatory cytokines as IL-10, IL-1ra or TGF-β (69). Via IL-8 and its mice analogous CXCL1 (also named KC) secretion, monocytes and macrophages are able to chemoattract neutrophils to the site of infection (70). All these cytokines then enable macrophages and monocytes to directly initiate different immune responses upon danger sensing (40).

1.2.9. Neutrophils

Neutrophils are also called polymorphonuclear neutrophil granulocytes (PMNs), as their nucleus is sectioned in several smaller lobules, which gives neutrophils their characteristic look and name. Their origin are hematopoietic stem cells in the bone marrow and they have a common precursor with mononuclear cells (40). PMNs are produced in large numbers daily. Via granulocyte colony stimulating factor (G-CSF) approximately $1\text{-}2x10^{11}$ cells are produced per day in an adult human in steady state conditions (71). These high numbers are needed, as neutrophils only have a half-life of approximately 11,4 h in circulation (67). Neutrophils that are not migrating to infection sites undergo apoptosis and are cleared by macrophages in the spleen or liver (40). During maturation, neutrophils produces three different types of granules for killing pathogens. These can be distinguished by the presence of either myeloperoxidase (MPO), lactoferrin or gelatinase. Because of these granules, neutrophils belong to the granulocytes and are distinguished from basophils and eosinophils, by their distinct granule compositions (71). For bacterial killing, neutrophils have a range of different mechanisms. They can phagocytose bacteria and kill them with the different antimicrobial substances in their granules, which are released into the phagosome by fusing with these granules. In addition, the granulocyte's content can also be released via degranulation into the surrounding area to kill bacteria outside the cell (70). Another mechanism is the release of the granulocyte's nuclear content together with chromatin, which then forms the so called neutrophil extracellular traps (NETs). These NETs were described in 2004 and enable the neutrophils to immobilize the invading bacteria to ensure high antimicrobial molecule concentrations for an efficient killing (72). Another important mechanism is the oxidative burst. The NADPH oxidase enzyme

complex as well as the release of MPO leads to the production of reactive oxygen species, either inside the phagosomes or in the surroundings of the neutrophil to effectively kill bacteria (73). Upon pathogen detection, cytokines like TNF or IL-1 are secreted at sites of infection, by macrophages and monocytes for example (68, 69), which increase the expression of adhesins on endothelial cells. Via chemokine, integrin and selectin dependent cascades, neutrophils are then recruited to these inflammation sites and pass the endothelial barrier (73). An important chemokine in this process is the chemokine CXCL8 also named IL-8 (40) and its mice analogous CXCL1 (70). Reaching the site of infection, neutrophils are then activated via PRRs like TLRs, cytokines (40) and anaphylatoxins, which stimulate the oxidative burst for example (74), allowing them to fulfill their tasks of killing bacteria with all the above mentioned mechanisms.

1.3. Anaphylatoxins and their receptors

The complement system is well known for the formation of the MAC on bacterial surfaces. This is especially important in fighting off *Nme*, to start lysis and thereby initiating their eradication. However as briefly mentioned above, there are many more functions in which the complement system is involved in. Research has revealed that more than 50 molecules are associated with the complement system, orchestrating the versatile processes. One of those very important functions is keeping the delicate balance of the immune system between wanted inflammation, upon pathogens detection, and the normal homeostasis (43).

1.3.1. Anaphylatoxins and their effector functions

An important fraction of the complement system are the so called anaphylatoxins, which mediate many different processes. The anaphylatoxins C3a and C5a are released as soluble mediators by the C3-convertase and the C5-convertase respectively (45). These anaphylatoxins are small polypeptides, with a size of 77 amino acids for C3a and a size of 74 amino acids for C5a (74). Anaphylatoxins regulate different processes as vasodilation, oxidative burst in neutrophils, macrophages and eosinophils or histamine release in mast cells and basophils. C3a triggers the production of IL-6 and TNF-a in monocytes and B cells and C5a additionally acts as a chemoattractant for neutrophils, macrophages, activated T- and B cells, basophils and mast cells and also takes part in tissue regeneration (74). By taking part in so many different processes, the anaphylatoxin's potency has to be controlled, which is accomplished by a constant degradation by carboxypeptidases. Cleaving off the C-terminal arginine end of C3a and C5a results in the complete destruction of activities in C3a-desArg and a greatly diminished activity in C5a-desArg (74).

1.3.2. Anaphylatoxin Receptors

All the different functions of C3a and C5a are initiated by binding of the anaphylatoxins to their respective anaphylatoxin receptors (ATRs) C3aR, C5aR1 or C5aR2. The C3aR detects the anaphylatoxin C3a, whereas C5a is detected by either C5aR1 or C5aR2, with a higher affinity for the first. As mentioned above, the des-arginated form C3aR-desArg is not active anymore, due to the fact, that C3aR can only bind the C3a form. In contrast to that, C5aR1 and C5aR2, can still bind the des-arginated form C5a-desArg, however with a much lower affinity than the C5a form (74). The ATRs belong to the superfamily of G-protein-couple receptors (GPCRs) and are nowadays classified as a complement peptide receptor family in the group of peptide binding receptors in class A of the GPCRs as stated in the GPCRdb database (75, 76). GPCRs

in general are involved in transmitting many different signals, which can lead to changes in cellular processes. GPCRs are characteristically comprised of seven transmembrane domains, their extracellular N-terminus and their intracellular C-terminus (77). Upon agonist binding to the receptor, a conformational change occurs, which results in the opening of the intracellular coupling interface. The activated GPCRs can then bind to G-proteins to start G-protein-dependent signaling, or are phosphorylated by G-protein-coupled receptor kinases (GRK) allowing the binding of arrestins, to conduct G-protein-independent signaling (78). The exact mechanisms underlying those two signaling pathways are still under investigation, especially as up to 40% of all pharmaceuticals are targeting GPCRs (79). Previously it was believed that phosphorylation of GPCRs and arrestin binding mainly hinders the G-protein binding, to dampen the signal transduction, this view has changed. More recent evidence suggest, that there are underlying mechanisms which are started via β-arresting binding, and that it is in fact a G-protein independent signaling mechanism (79). In fact, possibly through different GPCR binding sites and conformational changes, ß-arrestin can even initiate different pathways (78), for example via ERK1/2 (80). From the three ATRs only C3aR and C5aR1 transcode intracellular signaling via heterotrimeric G-proteins, whereas C5aR2 is constitutively uncoupled from G-proteins due to mutation in the DRY motive of the receptor (81). Nevertheless, as the view about β-arrestin in GPCR signaling has changed, the C5aR2 could still be able to signal via β-arrestin in addition to its role as a scavenger receptor for C5a (82). If the C5aR2 is an anti- or pro-inflammatory receptor is still topic of current research (83).

1.3.3. The ATRs C3aR, C5aR1 and C5aR2

All three ATRs are expressed on many different cells of myeloid or non-myeloid origin. This includes foremost monocytes/macrophages, mast cells, dendritic cells, basophils and eosinophils and microglia. Non-myeloid expression can be found in astrocytes, endothelial cells, epithelial cells, smooth muscle cells as well as in lung, liver, kidney, heart, muscle, ovary and testis tissues. In contrast, the expression on myeloid derived lymphocytes is still a matter of debate. C3aR was reported to be only expressed on activated T cells, whereas C5aR1 and C5aR2 might be expressed on naïve and activated T and B cells according to some groups and not according to others (74). Additionally, on dendritic cells only C5aR2 seems to be expressed (74). However, a huge difference can be found in the signaling of the three ATRs. C3aR and C5aR1 both express the DRY and NPXXY motifs, which are crucial elements for the binding of G-proteins and signal transduction. C5aR2 on the other hand has an leucin instead of an arginine in the DRY motif and lacks the tyrosine in the NPXXY motif, which is replaced by an

phenylalanine (75, 84). The lack of G-protein signaling gave rise to a controversial debate about C5aR2, whether it actually is a functional receptor or not (83). Originally it was believed that due to the lack of the above mentioned motifs, C5aR2 is not able to bind to G-proteins and no intracellular calcium mobilization, chemotaxis or MAPK signaling was observed in C5aR2 transfected cell lines upon C5a stimulation (81). Subsequently, it was shown that the C5aR2 is internalized upon ligand binding, adding up to the theory that the receptor is mainly involved in reducing the amount of C5a for C5aR1 and with the internalization capacity could be working as a scavenger receptor (85). Fact is, that the complete signaling of GPCRs is still not completely understood and that there is signaling dependent on G-proteins, but also signaling independent of G-proteins (78). This independent signaling mainly depends on β-arrestin. Originally β-arrestin was discovered as to be blocking signaling of G-proteins. More recent evidence suggests that there are several more pathways which are dependent on β-arrestin and that β-arrestin is responsible for desensitization of the receptor and its internalization in a clathrin dependent manner, which could also be shown for C5aR2 (79, 85). This process is believed to be not only responsible for recycling of the receptor, but also to be responsible to trigger further signaling mechanisms (78) and so it was shown, that there is recruitment of b-arrestin to C5aR2 in HEK cells expressing C5aR2 (86).

1.3.4. The role of ATRs in different diseases

With the anaphylatoxins being involved in so many different cellular processes, it is an important factor in many different pathologic conditions, such as cardiovascular diseases, diseases of the nervous system, asthma or sepsis upon bacterial infection (87). To each of those pathological conditions, the anaphylatoxins contribute differently. In some they are believed to take a more pro-inflammatory role in others a more anti-inflammatory role. In asthma for example, where the lung can react to foreign substances with an inflammatory condition, C3a and C5a are produced upon allergen detection. The anaphylatoxins can then act pro-inflammatory by chemoattraction of granulocytes through C5a, activation of granulocytes and mast cells and can stimulate smooth muscle contraction and mucus production (88). On the other side, C5aR signaling can be protective in the phase of allergen sensitization, by acting on the interplay between dendritic- and T-cells (89), highlighting the controversial involvements.

Another important role of the ATRs is their involvement in sepsis. One observation is that in IMD patients the amount of anaphylatoxin in their blood is strongly increased. The complement system is strongly activated, by the invading bacteria, resulting in a massive release of the anaphylatoxins C3a and C5a, which can have detrimental effects that are manifested in sepsis

(90). Similar dramatic effects upon strong C3a and C5a release could be seen in an animal model after cobra venom factor (CVF) administration (91). These findings raise the question, whether the C3aR, the C5aR1 or the C5aR2 might be good therapeutic targets for treating IMD in addition to the standard therapies (29). There is already data published by Rittirsch *et al.* for cecal ligation and puncture (CLP) induced sepsis, which showed a better outcome if C5aR1 or C5aR2 were blocked or absent in this murine model (92). In addition, Herrmann *et al.* showed, in an intra peritoneal (i.p.) meningococcal sepsis model, tremendous positive effects on the survival, when C5aR1 was absent or blocked (93). The roles of all three ATRs in a meningococcal sepsis model are now further investigated. Especially the roles of C5aR2 in IMD are still unclear and could shed further light on the overall function of this receptor and whether it is more pro- or anti-inflammatory in function (74).

1.4. *Nme* nasal colonization

Meningococci can only be found in humans and are passed on by droplets. Their first contact with their human host are the nasal cavities. In fact, 8-25% of the world's population are asymptomatic carriers of *Nme* in the mucosal sites of the nasopharyngeal tract (26). Mucosal sites are a special place for bacteria. On one hand they overtake important functions for the human host, especially in the gut, on the other hand the human body also has to protect itself from harmful bacteria. Several mechanisms are involved in this intricate interplay to ensure an environment where positive or neutral bacteria can be harbored, but possible pathogenic bacteria are fought off (94). As a first physical barrier, mucosal areas are therefore covered with mucus. The mucus layers contains nutritions for bacteria, but simultaneously keeps them at bay by a low viscosity, antimicrobial molecules and a constant mucociliary clearance (95). Another mechanism to control bacterial growth in the human body, is the limitation of important nutritions, like free iron with iron-binding proteins. In the mucus of the human nasopharynx the iron is mainly bound by lactoferrin (19). Furthermore, the most important part of the immune system in fighting off *Nme,* the complement system (47), is also active in mucosal sites (96). To overcome these different defense mechanisms, bacteria like meningococci have evolved a brought range of different strategies. For iron acquisition in mucosal sites, they express a lactoferrin receptor (19, 20). To overcome the complement system they express for example the factor H binding protein (57). Against the constant mucociliary clearance (95), meningococcal express a great variety of adhesins (97). Nevertheless, many of those adhesin might be sterically hindered by the expression of the capsule, as epithelial adhesion is increased in non-encapsulated *Nme in vitro* (98, 99). Therefore, Pili, which reaches beyond the capsule, and Opa, which can still bind to CEACAM-1 despite capsule expression, are believed to be the most important adhesins (100). During asymptomatic carriage, meningococci isolates may express a capsule or not, whereas in epidemic settings, meningococci mostly express a capsule (100). Adding to that, Johswich *et al.* could show that the expression of Opa and the subsequent binding to CEACAM-1 is sufficient for colonization of the nasopharynx and established thereby, an *in vivo* model to study this intricate interplay between meningococci and the human host (101). Next to the first physical barrier, the human immune system constantly surveils the mucosal surface, especially with dendritic cells and is able to elicit immune reactions if necessary (40). These inflammations then attract many more cells, such as neutrophils. In attraction and activation of these cells, the anaphylatoxins and their respective receptors play an important role, as shown in IMD (93). Therefore, it would be interesting to analyze, whether

the ATRs C3aR1, C5aR1 or C5aR2 would also have an impact on the mucosal colonization or clearing of *Nme*.

1.5. The CRISPR/Cas system and its possible involvement in mucosal colonization

The clustered regularly interspaced short palindromic repeats (CRISPR) loci and its CRISPR associated (Cas) proteins, in short CRISPR/Cas, compose an adaptive immunity system in prokaryotes, which is analogous to the RNA interference system in eukaryotes (102, 103). CRISPR/Cas loci were identified via genomic analysis, as being a common trait of most archaea and many bacteria. In 2002 it was first hypothesized that it could be a novel DNA repair system in prokaryotes (104). In 2006 Kira S. Makarova and colleagues theorized with *in silicio* findings that the CRISPR/Cas system could actually be a prokaryotic defense mechanism against phages and plasmids (103). This theory was then experimentally proven by Barrangou *et al.* in *Streptococcus thermophilus*, which acquired immunity against bacteriophages (105). Their experiments showed that the spacer sequences, located between the repeats in the CRISPR locus, are providing the specificity for the targeted phages and that the Cas proteins are providing the protection. This proofed the point that the CRISPR/Cas system is an adaptive immune system in prokaryotes (105).

1.5.1. CRISPR/Cas classification

Since then, there have been many advances in the CRISPR/Cas field and further analysis lead to the current classification of the CRISPR/Cas system into two classes, six types and several subtypes. Class 1 comprises the systems that have an effector molecule comprised of several subunits and class 2 the systems that have a single effector molecule. The different types are then further classified by the different involved Cas genes. The different classifications are not explained in more details, but just to give an example, the type I CRISPR/Cas system for example characteristically involves Cas3, has an effector module comprised of several sub-units and is therefore classified as a class 1 system. The well-known type II CRISPR/Cas system, on the other hand, is classified as a class 2 system, because it consists of a single unit as an effector module, which is Cas9 and relevant for this work (106). The type II CRISPR/Cas system is probably the most analyzed CRISPR/Cas system so far, as it is broadly known for its great potential for scientific research and future medical applications and is described now in more detail.

1.5.2. The type II CRISPR/Cas system

The type II CRISPR/Cas system was found in many bacteria and also in some *Nme* strains. The *Nme* strain MC58 was not found to encode genes for this system (107, 108), but it was found in the *Nme* strain 8013 (102, 108). As this work deals with type II CRISPR/Cas mutants from the strain 8013, this system is explained to some extend in more detail in this section. The first part of the CRISPR/Cas system is the acquisition of the protospacer, which is then incorporated as the spacer sequence and ultimately used for targeting foreign DNA. In this process involved are the highly conserved Cas1 and Cas2 molecules and depending on the different types and subtypes additional molecules. In type II CRISPR/Cas systems Cas9 plays an important role and depending on the subtype also Cas4 or Csn2. For identification of foreign DNA, type II CRISPR/Cas systems require a specific sequence, the protospacer adjacent motif (PAM). The incorporation of the protospacer as a spacer into the CRISPR array is then mediated by Cas1 and Cas2 (109, 110). The next part of the CRISPR/Cas cascade is the maturation of the CRISPR RNA (crRNA), for which, characteristically in CRISPR/Cas class 2 type II systems, only one effector molecule, the Cas9, is required. In the beginning of the crRNA maturation, the whole CRIPSR array is transcribed and this pre-crRNA contains all the spacer sequences, targeting the non-self DNA, and the small palindromic repeats. Unique to the type II CRISPR/Cas systems is also a gene for the small non-coding RNA *trans*-activating CRISPR RNA (tracrRNA). The *tracrRNA* gene, in short (*trc*), contains an in part complementary sequence to the repeat sequences in the pre-crRNA. Together with Cas9, the tracrRNAs can then bind to the pre-crRNA, forming a double-stranded RNA (dsRNA) at the repeat sites. This dsRNA is then cleaved by RNAse III and the matured tracrRNA:crRNA complex remains in bound to Cas9 for mediating the CRISPR/Cas effector functions (110-112). This process of crRNA maturation is characteristic for the type II CRISPR/Cas systems, however in *Nme*, which express the type II-C CRISPR/Cas system, this process is slightly different. In *Nme*, each repeat sequence contains a promoter and thereby can be transcribed individually. Due to those promoters, single crRNA can already be expressed, without the need for transcription of the whole CRISPR-array. Therefore, the maturation of the crRNA in *Nme* can be done independently of the tracrRNA and the RNAse III. However, for the Cas9 effector functions, the tracrRNA still needs to be attached to the crRNA (102). For ultimately targeting foreign DNA, the Cas9 tracrRNA:crRNA complex first searches for the specific PAM sites and upon finding, starts a more intense search for the complementary crRNA sequence (113). It was first predicted *in-silico*, that Cas9 contains two nucleases domains (103) and then shown *in-vitro*, that they are responsible for the effector function of Cas9 (114, 115). The RuvC-like nuclease domain

cleaves the anti-complementary strand and the HNH nuclease domain cleaves the complementary strand. Thereby Cas9 leaves blunt ends, three nucleotides away from the PAM, when targeting foreign DNA, which opens up the vast possibilities for this system in genomic engineering (114, 115).

1.5.3. The CRISPR/Cas system's possible role in virulence

The main function of the CRISPR/Cas system is the defense against mobile genetic elements (103, 105). In addition to this, further functions of the CRISPR/Cas system have been reported and are investigated. In *Myxococcus xanthus* for example, the Cas8c protein starts the expression of the *fruA* gene and is thereby involved in its sporulation process (116). In *Escherichia coli* the Cas1 protein must be involved in DNA repair, as without it, induced DNA damage was greatly increased (117). Other studies went even further and suggested that there could be a link between the CRISPR/Cas system and virulence traits. Louwen *et al.* discovered a reduced virulence in *Campylobacter jejuni* Δcas9 mutants (118). Sampson *et al.* postulated from experiments that the type II CRISPR/Cas system of *Francisella novicida* would be directly targeting the mRNA of an outer membrane protein, the bacterial lipoprotein (BLP) (119). However, as they were not able to reproduce their results, they had to publish an augmentation of their original paper (120). Nevertheless, they still stated that with an intact CRISPR/Cas system the BLP expression was reduced, but maybe not through a direct fashion (120). Additionally, they observed a reduced virulence of *Nme* in the absence of Cas9, when infecting alveolar cells (119, 120). Relating to that, Heidrich *et al.* conducted further experiments with a human nasopharynx cell line, showing that *Nme* were in fact reduced in their virulence traits, adhesion to these cells, without the type II CRISPR/Cas system (121). Another example is *Streptococcus agalactiae* in which Ma *et al.* saw a reduced virulence in *cas9* knock-out mutants. They ascribed this to a reduced degradation of the transcript from the transcriptional receptor *regR* in Δ*cas9* mutants (122). As the type II CRISPR/Cas system is described as targeting DNA (110), so far it was not clear how cas9 changes the transcriptional levels of BLP (119, 120) or the transcripts from *regR* (122). Recently it has been shown that Cas9 could also target RNA, which could explain the observed effects of cas9 on virulence traits (123, 124).

1.6. Aim of the study

Nme are gram-negative bacteria colonizing the human upper respiratory tract. These bacteria can become invasive and cause meningitis and fulminant sepsis. The mortality rate of meningococcal sepsis is, even with intensive care treatment, as high as 10% (28). This highlights the need for a better understand of fast progressing IMD and the need for developing new treatment options to support the standard IMD management. Most important in fighting off *Nme* infections is the complement mediated killing via MAC formation (47). During the complement cascades the anaphylatoxins C3a and C5a are released, which are detected via ATRs that are expressed on many different immune cells (74).

This work aims:

- to analyze the role of the ATRs C3aR, C5aR1 and C5aR2 in IMD.
- to highlight that next to the complement system also cellular components of the immune system are necessary in clearing of *Nme* infections *in vivo*.
- to test the targeting potential of the ATRs C3aR, C5aR1 and C5aR2 with therapeutics *in vivo* as well as *ex vivo*.
- to study possible underlying mechanisms that are changed in the absence of ATRs in neutrophils and macrophages, which could explain possible changes in IMD with impaired ATR signaling.
- to examine and compare possible murine findings in a human whole blood model by blocking the ATRs with different agents, to consider the specific host tropism of *Nme*.
- to evaluate a possible involvement of the complement system and the ATRs on meningococcal nasopharyngeal colonization in a murine huCEACAM-1 model.
- to analyze a possible involvement of the type II CRISPR/Cas system in mucosal colonization in a murine huCEACAM-1 model.

2. Materials and Methods

2.1. Materials

The following materials were either produced in house or bought externally.

2.1.1. Laboratory instruments

Table 1: Laboratory instruments

bio hazard bag	Bio Hazard Bag (Sarstedt, Nümbrecht, Germany)
BD FACSCalibur	BD FACSCalibur (BD, Franklin Lakes, USA)
centrifuges	Biofuge fresco (Heraeus, Hanau, Germany) Rotanta 460 R (Hettich, Tuttlingen, Germany)
ChemiDoc	ChemiDoc MP (Bio-Rad, Dreieich, Germany)
ELISA-Reader	Multiskan EX (Thermo Labsystems, Waltham, USA)
ELISA-Washer	NuncTM immuno Washer 12 (Nunc A/S, Roskilde, Denmark)
freezer	GGU 1500 (Liebherr, Bulle, Schweiz) (-22°C) ThermoForma -86C ULT Freezer (Thermo Scientific, (Waltham, USA) (-81°C)
fridges (4-7°C)	FKS 1800 (Liebherr, Bulle, Switzerland) KS 3160 (Liebherr, Bulle, Switzerland)
heating block	Thermochem (Liebisch, Bielefeld, Germany)
ice machine	AF 156 (Scotsman Ice Systems, Vernon Hills, USA)
incubators (37° C, 5% C02, 90% humidity)	B 6200 (Heraeus, Hanau, Germany) INC 246 (Memmert, Schwabach, Germany)
individually ventilated cages (IVC)	RAIR IsosystemTM (Lab Products, Seaford, USA)
magnetic stirring plate	MR 3001 (Heidolph, Schwabach, Germany) RCT (IKAMAG®, Staufen, Germany)
multi-channel pipette	VWR SignatureTM Ergonomic High-Performance 20-200 µl (VWR International, Darmstadt, Germany)
operation tools: anatomical scissors and forceps in different sizes	unknown producers (ordered through A. Hartenstein, Würzburg, Germany)
pH-Meter	Lab 850 (SI Analytics, Mainz, Germany)
photometer	CO8000 (WPA, Cambridge, UK)
pipette boy	accu-jet® pro (BRAND, Wertheim, Germany)
pipettes	Research® plus 0,5-10µl / 10-100µl / 100-1000µl (Eppendorf, Hamburg, Germany) Reference® 0,5-10µl / 10-100µl / 100-1000µl (Eppendorf, Hamburg, Germany) PIPETMAN P20 / P200 / P1000 (Gilson, Middleton, USA) Multipette® M4 (Eppendorf, Hamburg, Germany)
scales	ABT 120-5DM (Kern, Balingen-Frommern, Germany) PFB 6000-1 (Kern, Balingen-Frommern, Germany) P1200 (Mettler, Greifensee, Switzerland)
shaking plate	KL-2 (Edmund Bühler, Tübingen, Germany)

sterile working benches	LaminAir® HB 2472 (Heraeus, Hanau, Germany) MSC AdvantageTM 1.5 (Thermo Electron LED, Langenselboid, Germany)
timer	Timer SKT 338 N (Oregon Scientific, Tualatin, USA)
vortex	REAX 2000 (Heidolph, Schwabach, Germany)

2.1.2. Used materials

Table 2: Materials used in this work

48-well plates	Microtest Plate 48 Well (Sarstedt, Nümbrecht, Germany)
96-well-plates	Microtest Plate 96 Well, R (Sarstedt, Nümbrecht, Germany)
96-well-plates for ELISA	Microplate, 96 well, PS, U-bottom, MICROLON® 600, high binding, clear (Greiner Bio-One, Frickenhausen, Germany)
animal food	1314 TPF (Altromin, Lage, Germany)
blood agar plates (Columbia Agar + 5% sheep blood)	COS 90mm (bioMerieux, Marcy l´Etoile, France)
cell strainer	cell strainer EASYstrainer™, 40 µm (Carl Roth, Karlsruhe, Germany)
centrifugal filters	Amicon® Ultra 2 mL Centrifugal Filters for Protein Purification and Concentration (Merck Millipore, Merck KGaA, Darmstadt, Germany)
centrifugal tubes	15 ml / 50ml (Greiner Bio-One, Frickenhausen, Germany)
	Cellstar® Tubes 15 ml / 50 ml (Greiner bio-one, Frickenhausen, Germany)
cotton swabs	Wattestäbchen 15cm (Heinz Herenz, Hamburg)
cuvettes	Halb-Mikro-Küvette (Sarstedt, Nümbrecht, Germany)
disinfectant	Terralin® liquid (Schülke, Norderstedt, Germany)
disposal bag	Disposal bag (Sarstedt, Nümbrecht, Germany)
ELISA cover foil	Selbstklebende Polyester-Verschlussfolie für PCR-Platten (ordered through A. Hartenstein, Würzburg, Germany)
Falcon	FalconTM, 5 ml Polystyrene Round-Bottom Tube (BD, Franklin Lakes, USA)
(modified Thayer-Martin agar plates) GC-VCNT agar plates	36 g BD DifcoTm Chocolate Agar Base (GC Medium) (BD, Franklin Lakes, USA) solved in 1 L ddH20, autoclaved at 121°C. While cooling down the agar was enriched at approximately 50°C with the following ingredients: 10 ml sodium bicarbonate, 10 ml Kellogg´s supplement and 10 ml of VCNT Inhibitor. Additionally, kanamycin was added if needed. The agar was poured into petri dishes, dried at RT for 48 h and then stored at +4°C.
chocolate agar plates	GC-VCNT-plates + 1% Hemoglobin (BD Difco)

gloves	Peha-soft® nitril fino (Hartmann, Heidenheim, Germany)
mouth protection mask	Foliodress® mask Loop (Hartmann, Heidenheim, Germany)
needles	MicrolanceTM 3 26G x 3/8“ (BD, Franklin Lakes, USA)
	NEOJECT® 25G x 5/8“ (Dispomed, Gelnhausen, Germany)
Nitrocellulose membrane	Nitrocellulose membrane (GE HEaltcare, München, Germany)
parafilm	Parafilm M, 4IN. X 125 Ft. (Bemis, Neenah, USA)
petri dishes	Petrischalen 92x16mm (Sarstedt, Nümbrecht, Germany)
pipette tips (Dispenser)	Combitips advanced® 1ml / 5ml (eppendorf, Hamburg, Germany)
pipette tips (pipettes)	10µl / 200µl / 1000µl (Sarstedt, Nümbrecht, Germany)
plastic bowls	Reagent Reservoirs 50 ml (VWR International, Radnor, USA)
Plastic Inoculation loops	Impfschlinge 10 µl (Sarstedt, Nümbrecht, Germany)
reaction tubes	Eppendorf Tubes® 0,5ml / 1,5ml / 2,0ml / 5,0ml (eppendorf, Hamburg, Germany)
serological pipettes	Serological pipettes 5 ml/ 10 ml/ 25 ml (Sarstedt, Nümbrecht, Germany)
single use body protection cover	Einmalkittel orange (VE 100 Stück) (RMP-med, Dresden, Germany)
single use operation net	ordered through A. Hartenstein, Würzburg, Germany
single use syringes	1 ml TBC (Dispomed, Gelnhausen, Germany)
	1,6 ml S-Monovette® Hirudin (Sarstedt, Nümbrecht, Germany)
	5 ml SOFT-JECT® / 10 ml SOFT-JECT® (Henke Sass Wolf, Tuttlingen, Germany)
	50 ml INFUJECT® (Dispomed, Gelnhausen, Germany)
sterile filters	Filtropur S 0.2 (Sarstedt, Nümbrecht, Germany)

2.1.3. Chemical compounds

Table 3: Chemical compounds

10xPBS (sterile)	gibco® DPBS powder without calcium chloride and magnesium chloride (life technologies Corporation, Grand Island, USA)
SB290157	C3aR antagonist, SB290157 trifluoroacetate salt (Sigma Aldrich/Merck (Missouri, USA)
$A8^{\Delta 71-73}$	C5aR1 and C5aR2 antagonist, was kindly provided by Prof. Dr. Jörg Köhl (Institute for Systemic Inflammation Research, University of Lübeck, Lübeck, Germany)
aqua dest (ddH20)	Aqua B. Braun Ecotainer® (B. Braun, Melsungen, Germany)
Avidin-Peroxidase	Avidin-Peroxidase 1mg/ml (A3151-1MG) (Sigma Aldrich/Merck, Missouri, USA)

BHI-Bouillon	BD Bacto™ Brain Heart Infusion (BHI) (BD, Franklin Lakes, USA)
BSA	A1391,0100 Albumin Fraction V (pH 7,0) (PanReac AppliChem ITW Reagents, Darmstadt, Germany)
C5a	Purified Recombinant Mouse C5a (BD, Franklin Lakes, USA)
clodronate liposomes	Clodronate Liposomes (Clodronate Liposomes.com, Amsterdam, Netherlands)
compstatin Cp20	The compstatin Cp20 peptide (Ac-Ile-[Cys-Val-Trp(1-Me)-Gln-Asp-Trp-Sar-Ala-His-Arg-Cys]-mIle-NH_2) was kindly provided by Professor John D. Lambris (University of Pennsylvania School of Medicine, USA)
DHR123	Sigma Aldrich/Merck (Missouri, USA)
DMEM Medium	Dulbecco's Modified Eagle Medium (Life Technologies/ Thermo Fisher Scientific, Massachusetts, USA)
EDTA	EDTA disodium salt dihydrate BioChemica (AppliChem GmbH, Darmstadt, Germany)
ethanol	Ethanol Rotipuran® ≥99,8% p.a. (Carl Roth GmbH, Karlsruhe, Germany)
fetal calf serum (FCS)	Fetal calf serum (Thermo Fisher Scientific, Massachusetts, USA)
GC Medium	BD DifcoTm Chocolate Agar Base (GC Medium) (BD, Franklin Lakes, USA)
Glycerin (Propantriol)	Glycerin 86% Rotipuran® p.a. (Carl Roth GmbH, Karlsruhe, Germany)
Heparin	Heparin (5000 U/ml) (Biochrom AG, Berlin, Germany)
iron dextran	D8517 Iron-Dextran solution (Sigma Aldrich/Merck, Missouri, USA)
KBR-buffer	KBR-buffer (virion\serion, Würzburg, Germany)
Laemmli buffer	Laemmli Sample Buffer (BioRad, Feldkrichen, Germany)
paraformaldehyde	Paraformaldehyd reinst, DAC (Carl Roth GmbH, Karlsruhe, Germany)
PMA	Sigma Aldrich/Merck (Missouri, USA)
PMX205	C5aR1 antagonist, was kindly provided by Prof. Dr. Trent M. Woodruff (School of Biomedical Sciences, The University of Queensland, Brisbane, Australia)
PMX53	C5aR1 antagonist (Tocris Bioscience, Bristol, UK)
RPMI medium	RPMI medium (Thermo Fisher Scientific, Massachusetts, USA)
saline	0,9% Natriumchlorid 100ml (Fresenius Kabi, Bad Homburg, Germany)
sulfuric acid	Schwefelsäure 96% (Roth, Karlsruhe, Germany)
superagonist (WWGKKYRASKLGLAR)	C3aR agonist, was kindly provided by Prof. Dr. Trent M. Woodruff (School of Biomedical Sciences, The University of Queensland, Brisbane, Australia)
Tween 20	Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany)
VCNT Inhibitor	BBLTM VCNT Inhibitor 10ml (BD, Franklin Lakes, USA)
W-54011	C5aR1 antagonist (Merck Millipore, Massachusetts, USA)

2.1.4. Reaction kits used

Table 4: Reaction kits

Target	Kit
mouse CXCL/KC ELISA kit	DuoSet® ELISA Development System Mouse CXCL1/KC (R&D Systems, Minneapolis, USA)
mouse IL-6 ELISA kit	DuoSet® ELISA Development System Mouse IL-6 (R&D Systems, Minneapolis, USA)
TMB Substrate	TMB Substrate Kit, Pierce™ (Thermo Scientific, Rockford, USA)
Human C5a ELISA kit	DuoSet® ELISA Development System Human Complement Component C5a (R&D Systems, Minneapolis, USA)
Human IL-8 ELISA kit	DuoSet® ELISA Development System Human IL-8/CXCL8 (R&D Systems, Minneapolis, USA)
LEGENDplex™	LEGENDplex™ Mouse Inflammation Panel (13-plex) (BioLegend, San Diego, USA)

2.1.5. Antibodies used

Table 5: Antibodies used

a-CD11b	APC/Cy7 anti-mouse/human CD11b, clone M1/70 (BioLegend, San Diego, USA) FITC anti-mouse/human CD11b, clone M1/70 (BioLegend, San Diego, USA)
a-CD68	FITC anti-mouse, clone FA-11 (BioLegend, San Diego, USA)
a-ERK1/2	rabbit-anti-ERK1/2 #9102 (Cell Signaling, Cambridge, United Kingdom)
a-F4/80	PE anti-mouse, clone BM8 (BioLegend, San Diego, USA)
a-Ly-6G	APC anti-mouse Ly-6G Antibody, clone 1A8 (BioLegend, San Diego, USA)
a-phospho-ERK1/2	rabbit-anti-phospho-ERK1/2 #9101 (Cell Signaling, Cambridge, United Kingdom)
HRP conjugate	Goat-anti-rabbit-HRP conjugate (Jackson ImmunoResearch, Cambridge, United Kingdom)
isotype control antibody	FITC at IgG2b, κ Isotype Ctrl Antibody, clone RTK4530 (BioLegend, San Diego, USA)
purified anti-human C5L2	Purified anti-human C5L2 Antibody, clone 1D9-M12 (BioLegend, San Diego, USA)
purified anti-human CD88 (C5aR)	Purified anti-human CD88 (C5aR) Antibody, clone S5/1 (BioLegend, San Diego, USA)
RB6/8C5 depletion antibody	Rat-IgG2B anti-Ly6G and anti-Ly6C (the hybridoma cell line was kindly provided by Paul Allen, University Washington, School of Medicine, St Louis, USA)

2.1.6. In house produced liquids

Table 6: In house produced liquids

BHI	37 g BactoTm BHI is solved in 1L ddH20, 15 min autoclaved at 121°C
100 x Kellogg´s supplement	40 g glucose
	1 g glutamine
	2 mg thiamine pyrophosphate
	5 mg iron (III)-nitrate
	ad 100ml ddH20, stored at -20°C

2.2. Bacteria

2.2.1. Biosafety and storage of bacteria

All procedures with viable bacteria or sterile processes like tissue culture were conducted in a biosafety level 2 classified facility. Handling of meningococci was done with great care in class II laminar airflow cabinets. The used bacterial strains were stored in 30% Glycerin in BHI at -80 °C.

2.2.2. *Nme* strain MC58 and mutant

The meningococcal strain Mc58 was first isolated in 1983 in the United Kingdom (125-128). The used strain's designation is B: P1.7, 16-2: F1-5: ST-74 (cc32) and was kindly provided by Andrew Gorringe (Public Health United Kingdom). For experiments with *Nme* lacking a capsule, the capsule-deficient mutant from MC58, MC58Δ*csb*-GFP, expressing a green fluorescent protein, was used (129).

2.2.3. *Nme* strain 8013 and mutants

The meningococcal strain 8013 was isolated in France 1989. The used strains designation is: C: P1.21,26-2: F1-5: ST-177 (130). For intranasal infection experiments, to analyze a possible benefit of the CRISPR/Cas system on mucosal colonization, the following mutants of 8013 were used: Δ*cas9* (clone 1) and its complementary mutant, Δ*cas9* (clone 2) and its complementary mutant, Δ*trc* and its complementary mutant and the double deletion mutant Δ*rcoF1*Δ*rcoF/2* (ΔΔ*rcoF1/2*). Their generations are publicized by Nadja Heidrich and colleagues (121, 131). The mutants were kindly provided by Prof. Dr. Christoph Schoen.

2.3. Animals

2.3.1. General information

All animal experiments described in this dissertation, as well as animal holding and handling, were conducted in accordance to the German Animal Welfare Laws ("Tierschutzgesetz" and "TierSchVersVO") and approved by the Government of Lower Frankonia (Regierung von Unterfranken) in "Bayern" under the files "55.2-2532-2-165", "55.2 DMS 2532-2-93" and "55.2-2531.01 14/14". All efforts were made in order to minimize the suffering and distress of the animals.

2.3.2. Animal housing

Animals were kept in the animal facility of the Institute for Hygiene and Microbiology at the University of Würzburg in accordance to the "TierSchVersVO". Housing was done with a 12 h bright and dark cycle, at a relative humidity of 55 ± 10% and a temperature of 22-24 °C. *Ad libitum* accesses to standard rodent diet and water was ensured and all cages were equipped with enrichments. Daily visual checkups ensured the wellbeing of all mice. To ensure a high level of standard, guidelines in the institute were in place for regulating the animal care taking, as well as the hygiene concept of the animal facility. A high hygiene status was kept by personal and material barriers. To ascertain the specified pathogen free (SPF) status of the mice, quarterly testings of sentinel animals were performed, as recommended by the Federation for European Laboratory Animal Science Associations (132). From these testings, the mice can be classified as SPF and additional quarterly controls were conducted to the facility to test for unspecific pathogens. For the experiments, the mice were transferred to a system with individually ventilated cages to ensure personal safety. All animals in the experiments were on a C57BL/6J background.

2.3.3. Genotypes

C57BL/6J wild type (WT) mice were purchased from Charles-River or Envigo or bred in-house. The mouse strains lacking the anaphylatoxin receptors were on a C57BL/6J background. Mice from the genotype lacking the C3aR, with the full name: B6.129S4-*C3ar1*^{tm1RWe}, were termed *C3ar1*$^{-/-}$ (133). Mice from the genotype lacking the C5aR1, with the full name: B6.129S4-*C5ar1*^{tm1Cge}, were termed *C5ar1*$^{-/-}$ (134). The *C5ar1*$^{-/-}$ mice were initially provided by Prof. Dr. med. Andreas Klos and bred in the Institute for hygiene and microbiology. Mice from the genotype lacking the C5aR2, with the full name B6.129S4-*C5ar2*^{tm1Cge}, were termed *C5ar2*$^{-/-}$ (135). Mice from the genotype expressing human CEACAM1, which was backcrossed

for 10 generations onto a C57BL/6J background, were termed *huCCM1* (136). Mice from the genotype lacking C5, with the full name: B6.FVB-Hc^0, were termed *C5*$^{-/-}$ (93). The mouse lines *huCCM1/C3*$^{-/-}$, *huCCM1/C5*$^{-/-}$, *huCCM1/C3ar1*$^{-/-}$, *huCCM1/C5ar1*$^{-/-}$, *huCCM1/C5ar2*$^{-/-}$, were interbreeds from mice lacking the specific complement component or receptor (*C3*$^{-/-}$, *C5*$^{-/-}$, *C3ar1*$^{-/-}$, *C5ar1*$^{-/-}$ or *C5ar2*$^{-/-}$) and *huCCM1* mice, expressing human CEACAM-1.

2.4. *In vivo* Infections of mice with *Nme*

2.4.1. Inoculum preparation

The bacteria used in the experiments were plated onto blood agar plates, with a single usage inoculation loop, one day prior to the infection. Incubation was done at 37 °C, 5% CO_2 with saturated humidity levels. On the day of the infection, bacteria were transferred with a cotton swab and spread all over a fresh blood agar plate. This plate was incubated for another 4 h to obtain potent bacteria from the logarithmic growth phase. From the logarithmic growth plate, the bacteria were scrapped with a cotton swab and resuspended in BHI. This bacterial suspension was adjusted to an OD_{600} value of 1,0 using a spectrophotometer. At this OD and with this protocol, the concentration of bacteria in the suspension equals $1{,}5x10^9$ colony forming units (CFU)/ml. This was confirmed each time by colony counting of serial dilutions from the suspension, after incubation on Columbia blood agar plates. From this initial suspension, further dilutions were prepared in BHI, to reach the desired concentrations for the different experimental settings.

2.4.2. Intra peritoneal infection with *Nme* for sepsis induction

Sepsis was induced in mice via i.p. injection of *Nme* into the lower left ventral area. 6-8 week-old male mice were subjected to one dose of 200 µl of *Nme* inoculum, which contained 10^4 CFU/ml (a nonlethal dose of *Nme* used in the depletion experiments) or 10^5 CFU/ml (a lethal dose of *Nme* used to induce a fulminant sepsis in experiments with ATR knock-out mice and therapeutic targeting of ATR in WT mice) in BHI. At this time of infection, termed as time point “0 h”, each mouse was additionally injected with a dose of iron dextran, diluted in sterile isotonic saline at a concentration of 1:3,33, into the lower right ventral area, for a potent infection (137). At 12 hours after infection, mice were injected with a second dose of iron dextran, diluted in sterile isotonic saline at a concentration of 1:3,33, into the lower right ventral area. For the injections 26 G needles with 1 ml syringes were used.

2.4.3. Cellular Depletion *in vivo*

Before and after infection, mice were treated with different compounds to target or stabilize different immune cells. Neutrophils were targeted with the monoclonal rat IgG2B antibody RB6-8C5. *In vivo* binding of this antibody to Gr-1 leads to the depletion of neutrophils and eosinophils (138-140). Mice were injected i.p. with 250 µg of RB6-8C5 antibody at -24 h, 0 h, 24 h and 48 h. Macrophages were targeted with clodronate liposomes. Clodronate liposomes are specifically taken up by mononuclear phagocytes (monocytes/macrophages), resulting in their depletion (141). Mice were injected i.p. with 200 µl of clodronate liposomes 3 days and 12 h before infection. Mast cells were stabilized using cromolyn (142). Mice were injected i.p. with 100 mg/kg cromolyn starting 48 h before infection and then every 12 h before and after infection for mast cell stabilization. Control mice were injected i.p. at the same time points with 200 µl PBS.

2.4.4. Intranasal infection with MC58 *in vivo*

The inoculum for the intranasal infection was prepared as described under 2.4.1., with the exception that instead of BHI, the inoculum was prepared in 1 mM $MgCl_2$. Ten µl of a final concentration of 10^7 CFU/ml of *Nme* were then carefully given onto both nares drop by drop. Monitoring of mice after intranasal infection yielded no bacteremia or clinical disease manifestations. 1-, 3- and 14-days post infection, the mice were euthanized by CO_2 inhalation, to analyze the rate of *Nme* colonization in the upper airways. First a nasal wash was performed by opening up the trachea and flushing the upper airways with 200 µl PBS with 1 mM $MgCl_2$. The wash solution was then collected from the nares into a 1,5 ml reaction tube placed above the nose. Next, swabs from the nasal mucosa were taken. A polyester-tipped swab pre-wetted with PBS with 1 mM $MgCl_2$ was inserted into the opened up nasal cavities. The nasal mucosal samples were taken by thorough rubbing and then resuspended in 500 µl PBS with $MgCl_2$. All samples taken, the nasal wash, the resuspended mucosal tissue samples and remaining tissue samples on the swab were dispersed on modified Thayer-Martin agar plates, which are selective for *Nme*. The plates were then incubated at 37 °C, 5% CO_2 with saturated humidity levels overnight for enumeration of recovered *Nme* after intranasal infection.

2.4.5. Intranasal infection with 8013 and mutants

Inoculum preparation was done as described under 2.4.1. for 8013 WT and each of its under 2.2.3. described mutants. Then, the inocula were mixed in equal parts as indicated, so that the final inoculum contained 50% of 8013 WT and 50% of one of the mutants. Five µl of a final

concentration of $2x10^{10}$ CFU/ml of *Nme* were then carefully given dropwise onto both nares. Monitoring of mice after intranasal infection yielded no bacteremia or clinical disease manifestations. Three days post infection, the mice were euthanized by CO_2 inhalation, to analyze the colonization rate. First a nasal wash was performed by retrograde flushing the upper airways through a tracheal incision with 200 µl PBS with 1 mM $MgCl_2$. The wash solution was then collected from the nares into a 1,5 ml reaction tube placed above the nose. Next, swabs from the nasal mucosa were taken. A polyester-tipped swab pre-wetted with PBS with 1 mM $MgCl_2$ was inserted into the opened up nasal cavities. The mucosal samples were taken by rubbing and resuspended in 500 µl PBS with $MgCl_2$. All samples taken, the nasal wash, the resuspended mucosal tissue samples and remaining tissue samples on the swab were dispersed on GC+VCNT-agar plates, which are selective for *Nme*. The plates were then incubated at 37 °C, 5% CO_2 with saturated humidity levels overnight. On the next day, up to 100 colonies were transferred to GC+ kanamycin agar plates and simultaneously to GC without kanamycin agar plate. These plates were incubated at 37 °C, 5% CO_2 with saturated humidity levels overnight. The next day, the colonies on the plates with and without kanamycin were enumerated in order to determine the competitive index between the 8013 WT and each 8013 mutant strain.

$$competitive\ index = \frac{CFUs\ of\ 8013\ mutant\ strain}{CFUs\ of\ 8013\ WT\ strain}$$

2.4.6. ATR targeting *in vivo*

ATRs were *in vivo* targeted with different compounds to either block or activate them. The C3aR was blocked using a chemical compound named SB290157 (143). Mice were injected i.p. with 100 µl of SB290157 at a concentration of 1 mg/kg body weight. Injection time points were 12 h and 6 h before infection, concurrent with the infection and then every 12 h post infection. To activate the C3aR, the superagonist (WWGKKYRASKLGLAR) was used (144). Mice were injected i.p. with 100 µl of the C3aR superpotent peptide at a concentration of 1 mg/kg body weight. Injection time points were 3 h before, concurrent with the infection and 6 h, 12 h and then every 12 h post infection. The C5aR1 was specifically blocked *in vivo* with the circular PMX205 (145, 146). Mice were injected i.p. with 100 µl of PMX205 at a concentration of 3 mg/kg body weight. Injection time points were 12 h and 6 h before infection, together with the infection and then every 12 h post infection. The receptors C5aR1 and C5aR2 were simultaneously blocked with $A8^{\Delta 71\text{-}73}$ (147). Mice were injected i.p. with 200 µl of $A8^{\Delta 71\text{-}73}$ at a concentration of 88,7 µM. Injection time points were 6 h before infection, together

with the infection, 6 h and 12 h after infection and then every 12 h post infection. All reagents were freshly prepared and sterile filtered in the vehicle solution with 5% glucose. As a control, WT mice were injected i.p. with the vehicle solution 12 h and 6 h pre infection, together with the infection, 6 h and 12 h after infection and then every 12 h post infection.

2.4.7. Clinical monitoring

All mice were closely monitored in accordance to each specific animal experiment application (Tierschutzantrag) approved by the Government of Lower Frankonia (Regierung von Unterfranken). All mice were weighed, monitored and scored at specific time points throughout the experiments. In accordance to the German Animal Welfare Laws, mice were euthanized when reaching the humane endpoint or the experiment's end. Table 7 describes the specific points mice were given for weight loss, general condition, behavior and the clinical condition. These points where then summed up to the overall score for each mouse at each specific time point. This score specified the individual burden on the mice throughout the experiment. A minor burden was declared for a score of up to 9 points, a medium burden was specified in the range of 10 to 19 points and a high burden for a score above or equal to 20 points.

Table 7: Scoring scheme

score	symptoms/condition
	Body weight:
0	weight gain or body weight loss <3%
1	weight loss >3% to 5%
5	weight loss ≥5% to 10%
10	weight loss ≥10% to 20%
20	weight loss ≥20%
	General condition:
0	normal condition, no symptoms visible as described below
1	small aberrations from normal condition (for examples slightly frizzy fur)
5	slightly hunched posture, frizzy fur
10	strong hunched posture, dirty fur, unclean anus, smudgy eyes
20	severe dehydration, shakiness
	Behavior:
0	normal condition, no symptoms visible as described below
1	small aberrations from normal behavior
5	impaired motility
10	co-ordinational problems, self-isolation, lethargy
20	no defensive behavior shown upon picking up, unconsciousness
	Clinical condition:
0	normal condition, no symptoms visible as described below
1	small aberrations from normal clinical condition
10	breathing frequency changed by ±30%
20	breathing frequency changed by ±50%, impaired blood flow

2.4.8. Blinded study design

The experiments for the cellular involvement in meningococcal sepsis were done using a blinded study design. Therefore, treatments of mice were carried out by a different person than the monitoring. Thereby, it was ensured that the results were unbiased as to which substance could show an effect or not. Until the end of the experiments, no informational exchange occurred between these persons regarding the experimental setup and therefore these experiments can be considered unbiased.

2.5. Sample acquisition *in vivo*

2.5.1. Bacterial burden assessment *in vivo*

To assess the bacterial burden in mice after infection, tail vein blood was drawn at specific time points. The mouse tail was disinfected and carefully warmed under an infrared lamp to dilate blood vessels. The tail vein was punctured with a sterile 25 G syringe. Five µl of tail vein blood was drawn 3 h, 12 h and then every 12 h until the experimental end or upon reaching the humane endpoint. At the humane endpoint, additional heart blood was drawn with a sterile 25 G syringe. The upper ventral area of the mouse was disinfected before puncturing. Blood was taken from the heart with a 1 ml syringe placed on the needle. For prevention of clotting, 5 µl of tail vein or heart blood were diluted in 45 µl of PBS containing 5 U/ml heparin. From this suspension five serial dilutions with a dilution factor of 1:10 in PBS were made and 20 µl of each dilution was plated onto Columbia sheep blood agar plates. After an overnight incubation at 37 °C, 5% CO_2 with saturated humidity levels, the bacterial colonies for each dilution were enumerated, to determine the bacterial concentration *in vivo*.

2.5.2. Blood collection for cytokine measurements

For cytokine measurements, tail vein blood was drawn and collected. The mouse tail was disinfected and carefully warmed under an infrared lamp to dilate blood vessels. The tail vein was punctured with a sterile 25 G syringe. Ten µl of tail vein blood was drawn 12 h post infection and diluted in 90 µl PBS containing EDTA for an end concentration of 5 mM EDTA. These 100 µl were stored on ice at first and then centrifuged at 10.000 G for 5 min at 4°C to separate the plasma from the cellular components of the blood. The supernatant was transferred to new tubes and stored at -80 °C for ELISA measurements.

2.6. ELISA measurements

2.6.1. DuoSet® ELISA-Kit measurements

Cytokine measurements were done in plasma of infected mice or plasma from infected murine or human whole blood using DuoSet® ELISA kits. The kits were used as described by the manufacturer with the following additions. The measurements were done at RT in 96 well plates, which were sealed with a seal during incubation times. For safety reasons, the ELISAs, with possible infectious material, were performed under a sterile work bench. Washing was done with 1xPBS/0,05% Tween. Blocking was done with 1xPBS/0,1% BSA. For analysis with an ELISA reader, TMB substrate was used and the reaction was stopped with 1 M sulfuric acid H_2SO_4.

2.6.2. Bead-based LEGENDPlex™ assay

Cytokine measurements were done in plasma of infected mice using the Bead-based LEGENDPlex™ assay. The LEGENDPLEX™ kit was used as described by the manufacturer with the following addition. Instead of matrix C, assay buffer with gentamycin was used. The kit procedures were done in v-bottom 96 well plates. For safety reasons the kit was used under a sterile work bench.

2.7. Cellular response measurements in mouse whole blood

2.7.1. Collection of mouse whole blood

Mice of the different genotypes were euthanized via CO_2 inhalation. The upper ventral area of these mice was disinfected and via heart punctuation mouse whole blood was collected. For collection, hirudin was chosen as anticoagulant at a concentration of 525 antithrombin units/ml, as hirudin does not interfere with complement nor the viability of *Nme* (148).

2.7.2. Oxidative burst measurements in mouse whole blood

Collected mouse whole blood was infected with 10^7 CFU/ml of *Nme*. As a positive control 100 nM PMA was added to whole blood samples and as a negative control PBS. The treated samples were incubated for 15 min at 37 °C rotating. To measure the oxidative burst, dihydrorhodamine 123 (DHR123) was added to each sample at a concentration of 20 µg/ml. The oxidative burst measurement with DHR123 in human and murine whole blood models via FACS analysis was established by Emmendörffer and colleagues (149). The samples were incubated for 1 h at 37 °C rotating. After the incubation time, the samples were placed on ice and stained with anti-mouse Ly6G-APC (Clone: 1A8; BioLegend) for 30 min. Next the

erythrocytes were lysed hypotonically with aqua dest, which was stopped by adding 10x PBS. The samples where then washed with cold PBS. The washed samples were resuspended in 4% formaldehyde and fixated at RT for 30 min without exposure to light. For assessing the oxidative burst, the samples were measured on a BD FACSCalibur and flow cytometry data were analyzed via FlowJo. An FSC/SSC gate excluded cell clusters and neutrophils were isolated via a gate for Ly6G-APC^{high} cells. The mean fluorescence intensity (MFI) for FL1-H of these cells was then displayed in a graph, which represents the intensity of oxidized DHR123 and thereby is a direct measurement of the oxidative burst in mouse whole blood neutrophils. In the samples displayed, the signal of the isotype PBS control was subtracted from the PBS-, infected- and PMA samples, to correct for a slight FL1-H shift of cells isolated from *C5ar2*$^{-/-}$ cells, due to low level GFP expression in these mice.

2.7.3. Degranulation measurements in mouse whole blood

Collected mouse whole blood was infected with 10^7 CFU/ml of *Nme*. As a positive control 100 nM PMA was added to whole blood samples and as a negative control PBS. The treated samples were incubated for 60 min at 37 °C rotating. The samples were then placed on ice and stained for further analysis via flow cytometry. The whole blood samples were stained with anti-mouse Ly6G-APC and anti-mouse/human CD11b-FITC or as a control, with anti-mouse Ly6G-APC and FITC labeled rat IgG2b, κ Isotype Ctrl Antibody for 30 min on ice. Next the erythrocytes were lysed hypotonically with aqua dest, which was stopped by adding 10x PBS. The samples where then washed with cold PBS. The washed samples were resuspended in 4% formaldehyde and fixated at RT for 30 min without exposure to light. The samples were measured on a BD FACSCalibur and flow cytometry data were analyzed via FlowJo. An FSC/SSC gate excluded cell clusters and neutrophils were isolated via a gate for Ly6G-APC^{high} cells. The MFI for FL1-H of these cells was then displayed in a graph, which represents the amount of CD11b expression and thereby is a direct measurement of the degranulation in mouse whole blood neutrophils. In the samples displayed, the signal of the isotype control was subtracted from infected- and PMA samples, to correct for a slight FL1-H shift of cells isolated from *C5ar2*$^{-/-}$ cells, due to low level GFP expression in these mice.

2.7.4. Phagocytosis measurements in mouse whole blood

Collected mouse whole blood was infected with 10^8 CFU/ml of GFP-expressing *Nme*. As a negative control PBS was added to the whole blood. The treated samples were incubated for 60 min at 37 °C rotating. The samples were then placed on ice and stained for further analysis via flow cytometry. The whole blood samples were stained with anti-mouse Ly6G-APC

antibody for 30 min on ice. Next the erythrocytes were lysed hypotonically with aqua dest, which was stopped by adding 10x PBS. The samples where then washed with cold PBS. The washed samples were resuspended in 4% formaldehyde and fixated at RT for 30 min without exposure to light. The samples were measured on a BD FACSCalibur and flow cytometry data were analyzed via FlowJo. An FSC/SSC gate excluded cell clusters and neutrophils were isolated via a gate for Ly6G-APC^{high} cells. The percentage of GFP positive PMNs was then displayed in a graph, which represents the GFP-expressing *Nme* and thereby is a direct measurement of the phagocytosis of *Nme* in mouse whole blood PMNs. In the samples displayed, the background signal difference between WT and *C5ar2*$^{-/-}$ neutrophils was subtracted from the PBS- and infected *C5ar2*$^{-/-}$ neutrophil samples, to correct for a slight FL1-H shift of cells isolated from *C5ar2*$^{-/-}$ cells, due to low level GFP expression in these mice.

2.7.5. *Nme* survival assessment in mouse whole blood

Collected mouse whole blood was infected with 10^6 CFU/ml of *Nme* or as a negative control PBS was added. Dilutions of the samples were plated onto blood agar plates at 0 h and 4 h post infection. The amount of bacteria, present at these time points, was assessed after overnight incubation at 37 °C and saturated humidity, via colony enumeration and plotted for assessment of *Nme* survival in mouse whole blood.

2.8. ERK1/2 phosphorylation measurements

2.8.1. Generation of bone marrow derived macrophages

WT, C5ar1 or C5ar2 knock-out mice were euthanized. Femurs and tibias were extracted and disinfected for 1 min in 70% ethanol. The bone ends were cut off and the bone marrow cells were flushed out with 15 ml PBS. Cell aggregates were separated by a 25 G needle passage. Possible bone fragment remains were removed with a 40 µm cell strainer. The suspension was centrifuged at 1000 g for 5 min followed by discarding the supernatant and hypotonic lysis of erythrocytes. The cells were then washed with 30 ml of PBS and again pelleted at 1000 g for 5 min. For macrophage differentiation the cells were resuspended in DMEM with 10% fetal calf serum and 10% L929 conditioned medium (150). 10^6 cells were seeded into each well of a 48-well plate. The medium was exchanged 3- and 6-days post seeding and at day 8, macrophage differentiation was determined by flow cytometry analysis for CD68, CD11b and F4/80 expression.

2.8.2. Western blot analysis for ERK1/2 phosphorylation

Western blot analysis of differentiated macrophages was done 8 days post bone marrow cell isolation. The cells were washed with PBS and KBR-buffer (10 mM HEPES, 125 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ at pH 7,3) containing 0,1% BSA was added. Thirty min later the macrophages were stimulated with 10 nM mouse C5a, 10^8/ml heat-inactivated *Nme,* C5a together with *Nme* or with 1 µM PMA. After an incubation time of 5-, 15- or 30 min the supernatant was taken out and the cells were lysed directly with 1x Laemmli buffer. The samples were then boiled for 10 min. Sample analyzing was done on 10% polyacrylamide gel, followed by transfer onto nitrocellulose membranes. These were then probed with rabbit-anti-phospho-ERK1/2 or rabbit-anti-ERK1/2 antibodies. Next goat-anti-rabbit HRP conjugate was used and the enhanced chemiluminescence was imaged on a ChemiDoc. Band intensities were measured with ImageLab (BioRad) and for the analysis, the band intensities were normalized by dividing the intensity of the phosphor-ERK band through the designated relative intensity of the total-ERK band.

2.9. Cellular response measurements in human whole blood

2.9.1. Collection and treatment of human whole blood

Via venipuncture human whole blood was drawn from healthy donors. For collection, hirudin monovettes were used. For analysis of the cellular responses in human whole blood in dependence on the anaphylatoxin receptors, the drawn whole blood was incubated for 10 min with one of the following reagents. To activate C3aR with 1 µg/ml C3aR-SAG, to inhibit C3aR with 200 µM SB29015, to block C5aR1 with 0,1/1/5/10 µM PMX53 or 3/30/300 nM W-54011, to simultaneously block C5aR1 and C5aR2 with 350 nM $A8^{\Delta 71\text{-}73}$, to block the complement completely at the C3-convertase with 30 µM compstatin Cp20, to block C5aR2 with 50 µg/ml C5aR2-antibody or as a negative control with RPMI. To remove sodium azide in the C5aR2-antibody, the antibody was diluted in PBS several times and each time concentrated again with Amicon Ultra centrifugal devices, with a cutoff at 3 kDa. The human whole blood samples were then incubated for 10 min at 37° C rotating.

2.9.2. Sample preparation for ELISA measurements in human whole blood

Collected human whole blood was infected with 10^6 CFU/ml of Nme or with RPMI as a negative control and incubated for 1,5 h at 37 °C rotating. Next, the samples were mixed in equal parts with ice-cold PBS containing 40 mM EDTA. These samples were then centrifuged

at 10.000 G for 5 min at 4°C to separate the plasma from the cellular components of the blood. The supernatant was transferred to new tubes and stored at -80 °C for ELISA measurements.

2.9.3. *Nme* viability tests in human whole blood with reagents

For *Nme* viability tests different concentrations of the reagents (C3 at 3/30/150 µM; C3aR-SAG at 0,1/2/10 µM; SB290157 at 2/20/200 µM; C5aR2-antibody at 0,1/1/10 µg/ml; $A8^{\Delta 71\text{-}73}$ at 3,5/35/350 nM) were used. The collected human whole blood was infected with 10^6 CFU/ml of Nme or with RPMI as a negative control and incubated for 30 min at 37 °C rotating. Next the samples were plated onto Columbia sheep agar plates in serial dilutions. After overnight incubation at 37 °C, 5% CO_2 with saturated humidity levels the CFUs were enumerated.

2.9.4. Oxidative burst measurements in human whole blood

Collected human whole blood was incubated with 20 µg/ml DHR123 for 10 min, before the treatment with the reagents listed under 2.8.1. The treated samples were infected with 10^7 CFU/ml of Nme or with RPMI as a negative control and incubated for 1 h at 37 °C rotating. Next the erythrocytes were lysed hypotonically with aqua dest, which was stopped by adding 10x PBS. The samples where then washed with cold PBS. The washed samples were resuspended in 4% formaldehyde and fixated at RT for 30 min without exposure to light. For assessing the oxidative burst, the samples were measured on a BD FACSCalibur and flow cytometry data were analyzed via FlowJo. Via an FSC/SSC gate, the distinct human neutrophil population was gated for. The MFI for FL1-H of these cells was then displayed in a graph, which represents the intensity of oxidized DHR123 and thereby is a direct measurement of the oxidative burst in human whole blood neutrophils.

2.9.5. Degranulation measurements in human whole blood

Collected and with the reagents listed under 2.8.1 treated human whole blood was infected with 10^7 CFU/ml of *Nme*. As a negative control RPMI was added to the whole blood. The samples were then incubated for 1 h at 37 °C rotating. The samples were then placed on ice and stained for further analysis via flow cytometry. The whole blood samples were stained with anti-human Ly6G-APC and anti-mouse/human CD11b-FITC or as a control, with anti-mouse Ly6G-APC and FITC labeled rat IgG2b, κ Isotype Ctrl Antibody for 30 min on ice. Next the erythrocytes were lysed hypotonically with aqua dest, which was stopped by adding 10x PBS. The samples where then washed with cold PBS. The washed samples were resuspended in 4% formaldehyde and fixated at RT for 30 min without exposure to light. The samples were measured on a BD FACSCalibur and flow cytometry data were analyzed via FlowJo. Via an FSC/SSC gate, the

distinct human neutrophil population was gated for. The MFI for FL1-H of these cells was then displayed in a graph, which represents the amount of CD11b expression and thereby is a direct measurement of the degranulation in human whole blood neutrophils.

2.9.6. Phagocytosis measurements in human whole blood

Collected and with the reagents listed under 2.8.1 treated human whole blood was infected with 10^8 CFU/ml GFP-expressing *Nme* or RPMI was added as a negative control. The samples were incubated for 60 min at 37 °C rotating. The samples were then placed on ice. Next the erythrocytes were lysed hypotonically with aqua dest, which was stopped by adding 10x PBS. The samples where then washed with cold PBS. The washed samples were resuspended in 4% formaldehyde and fixated at RT for 30 min without exposure to light. The samples were measured on a BD FACSCalibur and flow cytometry data were analyzed via FlowJo. Via an FSC/SSC gate, the distinct human neutrophil population was gated for. The percentage of GFP positive PMNs was then displayed in a graph, which represents the GFP-expressing Nme and thereby is a direct measurement of the phagocytosis of Nme in human whole blood neutrophils.

2.9.7. Statistics

The data in this work were statistically analyzed with Prism from GraphPad. Analysis of survival curves were done with the Mantel-Cox test. Statistical evaluation of data from more than two cohorts with parametric data (e.g. cytokine concentration) were done with one-way ANOVA using the Dunnett's *post hoc* test, when comparing treatments of genotypes to the corresponding controls, or the Bonferroni's *post hoc* test, when comparing all cohorts. The Kruskal-Wallis test was applied for non-parametric data (e.g. scoring data) from multiple groups using the Dunnett's *post hoc* test. Statistical analysis of data from two cohorts was done using the two-tailed Student's T-test for parametric data. Nonparametric data or data showing no normal distribution (according to Kolmogorov-Smirnov test) of two cohorts were compared using the Mann-Whitney test. To achieve normal distributed data from bacteremia analyses, these values were log-transformed. When log-transformation was done, the according graphs are plotted accordingly. Differences in results were declared as being significant at $P < 0,05$. Ns stands for significant; * stands for $P < 0,05$; ** stands for $P < 0,01$; *** stands for $P < 0,001$; **** stands for $P < 0,0001$.

3. Results

3.1. Cellular involvement in meningococcal sepsis

3.1.1. Depletion of monocytes/macrophages or neutrophils increases the lethality of meningococcal infections

The complement system is very important in fighting off meningococcal diseases. Studies showed a strong increase in meningococcal infections in humans with deficiencies in the alternative pathway, the complement factor C3, which is important for all three pathways, or the factors required for assembling the MAC (47). Such increased incidence rates in individuals with complement defects are also known for infections with *Streptococcus pneumoniae* (*S. pneumoniae*) or *Haemophilus influenza* (*H. influenza*). Those two bacteria can, like *Nme*, also cause meningitis or fulminant sepsis (47). Next to the humoral immunity also cellular defense mechanisms play an important role in fighting off bacterial infections. Whereas cellular responses in *H. influenza* infections seem to be impaired (151), cellular responses in *S. pneumoniae* infections are important in clearing the infection (152). Seeing these adverse cellular effects in infections with those two encapsulated bacteria, it would be interesting to analyze whether cellular responses might also play an important role in meningococcal infections.

Therefore, *in vivo* experiments were designed in which different cell-types, that play an important role in clearing bacteria as a first line of defense, are depleted in WT and *C5ar1*$^{-/-}$ mice. In order to be able to analyze their direct contribution to *Nme* infections, mice were injected with a nonlethal dose of 10^4 CFU of *Nme*. Cells that are important as a first line of defense and respond to the stimulation by released anaphylatoxins are mast cells, neutrophils and macrophages/monocytes (74). Mast cells are activated via C3a and C5a stimulation, which was shown on human skin mast cells by El-Lati and colleagues in 1994 (153) and in order to block these cells, mast cells were stabilized with cromolyn *in vivo* (142). As a first line of defense, especially phagocytic cells like neutrophils, monocytes or macrophages trigger different defensive processes, when detecting pathogens (154). Neutrophils can be depleted via injection of the monoclonal antibody RB6-8C5 (155) and macrophages/monocytes were simultaneously depleted using clodronate liposomes, which are specifically taken up by phagocytic mononuclear cells (141).

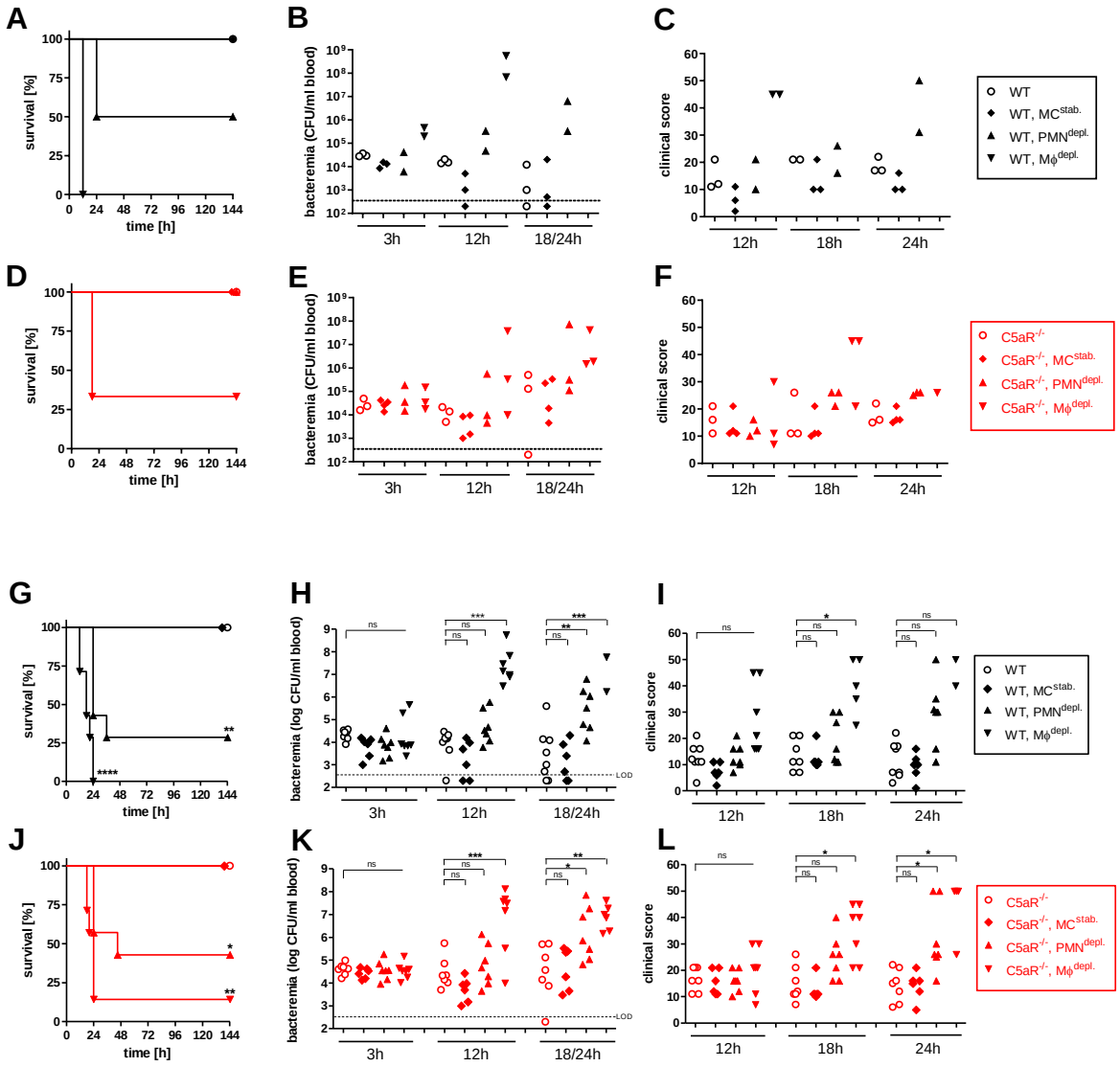

Figure 1: Differential impact of cell populations on experimental induced sepsis *in vivo*. **A-F** depict the data points acquired for this thesis from depletion experiments in WT and *C5ar1*$^{-/-}$ mice. Those data points are displayed in **G-L** in combination with the results from experiments done by Johannes Herrmann, published in his thesis in 2019 (156). The combined results in figure (Fig.) 1 **G-L** were published in the paper Herrmann *et al.* (93) and are modified depicted in this thesis. WT and *C5ar1*$^{-/-}$ mice were injected i.p. with a non-lethal dose of 10^4 CFU of Mc58 per animal. Iron dextran was administered i.p. at 0 h and 12 h post infection. Mast cell stabilization was done by injection of cromolyn starting 24 h pre-infection and then in a 12 h rhythm. Monocytes/macrophages were depleted via injection of clodronate liposomes 72 h and 12 h pre-infection. Neutrophils were targeted with injections of the monoclonal antibody RB6/8C5 every 24 h pre-infection, at time point 0 and 24 h and 48 h post infection. All mice were closely monitored for the duration of the experiment and euthanized upon reaching the humane endpoint. Blood samples were obtained 3 h and then in 12 h intervals post infection or upon reaching the humane endpoint, for assessing the bacterial burden of each mouse. The survival rates of the mice from the different groups are displayed for WT mice in **A** and **G** and for *C5ar1*$^{-/-}$ mice in **D** and **J**. **B** and **H** show the bacterial burden for WT mice and **E** and **K** for *C5ar1*$^{-/-}$ mice. The clinical score is depicted in **C** and **I** for WT mice and in **F** and **L** for *C5ar1*$^{-/-}$ mice. 500 CFU/ml was the limit of detection in **B**, **E**, **H** and **K**. ns, not significant;

*, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; in Mantel-Cox test for the survival rates with the control group as comparator, in one-way ANOVA with Bonferroni's *post hoc* test for the bacterial burden and in Kruskal-Wallis with Dunnett's *post hoc* test for the clinical score.

In the cellular depletion or stabilization experiments, both untreated control groups survived the nonlethal dose of *Nme* (Fig. 1G and J). Those mice completely cleared the bacterial infections and reduced their clinical scores during the course of the experiments. Cromolyn, which stabilizes mast cells, did not decrease the survival rate in either WT or *C5ar1*$^{-/-}$ mice (Fig. 1G and J). Additionally, there was also no change in the bacterial burden (Fig. 1H and K) or the clinical score (Fig. 1I and L) upon mast cell stabilization in either WT or *C5ar1*$^{-/-}$ mice, compared to untreated animals. WT or *C5ar1*$^{-/-}$ mice lacking neutrophils were more susceptible to this otherwise nonlethal dose of *Nme* and more than half of the mice in each group succumbed to the IMD. A significant increase in the bacterial burden could also be measured in both groups 18/24 h post infection. A trend to an increased clinical score was observable, but only significantly different 18/24 h post infection in *C5ar1*$^{-/-}$ mice. Depletion of monocytes and macrophages actually had a very severe impact on the outcome of the sepsis. Without monocytes and macrophages all WT mice and almost all *C5ar1*$^{-/-}$ mice reached the humane endpoint within the first 24 h (Fig. 1G and J). This outcome was also displayed in a strong significant increase of *Nme* in both animal groups, already as early as 12 h post infection (Fig. 1H and K). Likewise, the clinical score was increased from 12 h post infection onwards (Fig. 1I and L). Summarized, neutrophils and monocytes/macrophages also play an important role in fighting off IMD next to the complement system. Additionally, the experiments also showed that those protective mechanisms are not dependent on C5aR1, as those mice showed almost the same results in these depletion experiments.

3.1.2. Targeting C5aR1 in human whole blood reduced cytokine level of IL-8

Blockade of C5aR1 increased survival of mice in a murine meningococcal sepsis model (93). As contribution of that study, it was assessed in this work, if blockade of C5aR1 would result in similar outcomes in human whole blood infection with *Nme*. Therefore, the optimal concentration of the C5aR1 antagonists had to be found. Therefore, different concentration of the C5aR1 antagonists PMX53 and W-54011 were given to hirudin anticoagulated human whole blood (Fig.2). For each antagonist three different concentrations were tested. To measure the impact of the different concentrations and substances on the human whole blood, ELISA measurements for IL-8 were performed. The control samples were either incubated without bacteria (empty bar) or without any antagonist (filled bar) and display the basic IL-8 levels in the blood samples. The treatment with PMX53 and W-54011 yielded a significant reduction in

all three concentrations compared to the control sample, without any antagonist, showing that the blockade of C5aR1 also has a strong impact in human whole blood, letting one speculate a possible application in humans. The strongest effects of the antagonists on IL-8 could be observed with the highest concentrations of 10 µM for PMX53 and 300 nM for W-54011. Therefore, these concentrations were used for further analysis on the impact of C5aR1 blockaded in human whole blood, as published in Herrmann *et al.* (93).

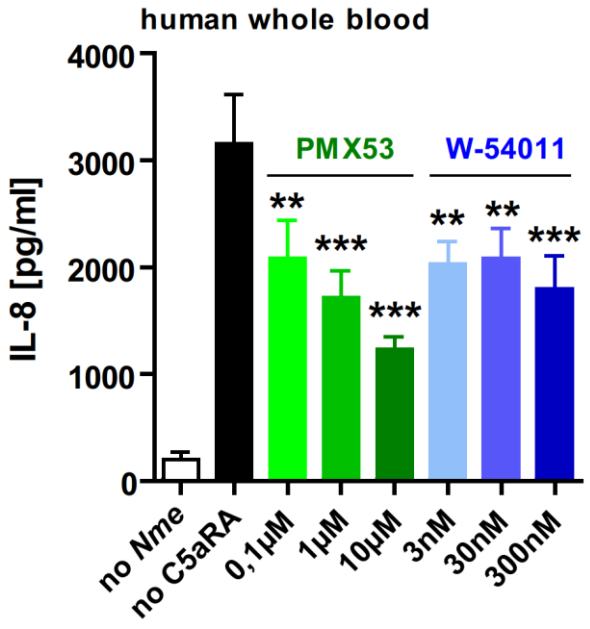

Figure 2: IL-8 measurement in human whole blood with blockade of C5aR1
To analyze the impact of C5aR1 blockade on the release of different cytokines, the needed concentration of C5aR1 antagonists was determined with this experiment. Using hirudin coated blood collection tubes, human whole blood was drawn from healthy donors and incubated with different concentrations of one of the two C5aR1 antagonists PMX53 or W-54011. The whole blood samples were infected with 10^6 CFU/ml of *Nme* MC58. The controls were either incubated without bacteria (empty bar) or without the C5aR1 antagonist (filled bar). IL-8 concentration was measured with ELISA. (means ± SEM; n = 5 donors). ns, not significant; **, P<0,01; ***, P<0,001; in one-way ANOVA using Dunnett's multiple *post hoc* test with the WT samples as a reference. The data were published in Herrmann *et al*.; 2017; mBio (93).

3.2. *In vivo* - The roles of the three ATRs in meningococcal sepsis

3.2.1. C3aR expression is important for IMD, whereas the lack of C5aR1 or C5aR2 is beneficial for the outcome

It is well established that the complement system and especially the MAC formation are the most important players in fighting off meningococcal infections (47). One part of the complement system however, the release of anaphylatoxins, has not been of high attention until recent years. Assembly of the C3- and C5-convertases leads to the release of the anaphylatoxins C3 and C5a and it is known that they are highly abundant in meningococcal sepsis (93). Anaphylatoxins are recognized by three different ATRs: C3aR, C5aR1 and C5aR2, which are expressed on a multitude of cell types and are involved in many different processes. For example are C3a and C5a involved in cytokine secretion, are capable of activating oxidative burst in neutrophils and macrophages and on top of that can C5a chemoattract neutrophils and macrophages (74). These versatile functions are mainly associated to the G-protein coupled receptors C3aR and C5aR1, but there are studies suggesting that also C5aR2 might have a signaling function via β-arrestin (86). This ultimately raises the question, what the role of these three ATRs and especially of the C5aR2 might be in meningococcal sepsis.

In order to analyze their direct impact on meningococcal sepsis, experiments with *C3ar*$^{-/-}$, *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ knock-out mice were conducted. Mice cohorts were injected with 10^5 CFUs of *Nme*/mice, which is a lethal dose for WT mice in these experimental settings. Three different parameters were compared: survival rate, bacterial burden and clinical scoring, between the four different mouse strains. For the bacterial burden, tail vein blood was drawn from each mouse 3 h, 12 h, 24 h, 36 h and 48 h post infection or when a mouse reached the humane endpoint. An additional heart whole blood sample was drawn upon euthanasia or at the end of the experiment. Using this lethal dose of *Nme* resulted in a survival rate of less than 10% in the WT reference group (Fig. 3A). Similar to that, mice lacking the C3aR also showed a survival rate of less than 10%, but those mice reached the humane endpoint significantly earlier (Fig. 3A). This intensified disease progression in *C3ar*$^{-/-}$ mice was also displayed in a significantly increased clinical score 18 h post infection. (Fig. 3B). The bacterial burden in those mice seemed to be slightly increased 12 h and 18 h post infection, but without significant levels (Fig. 3C).

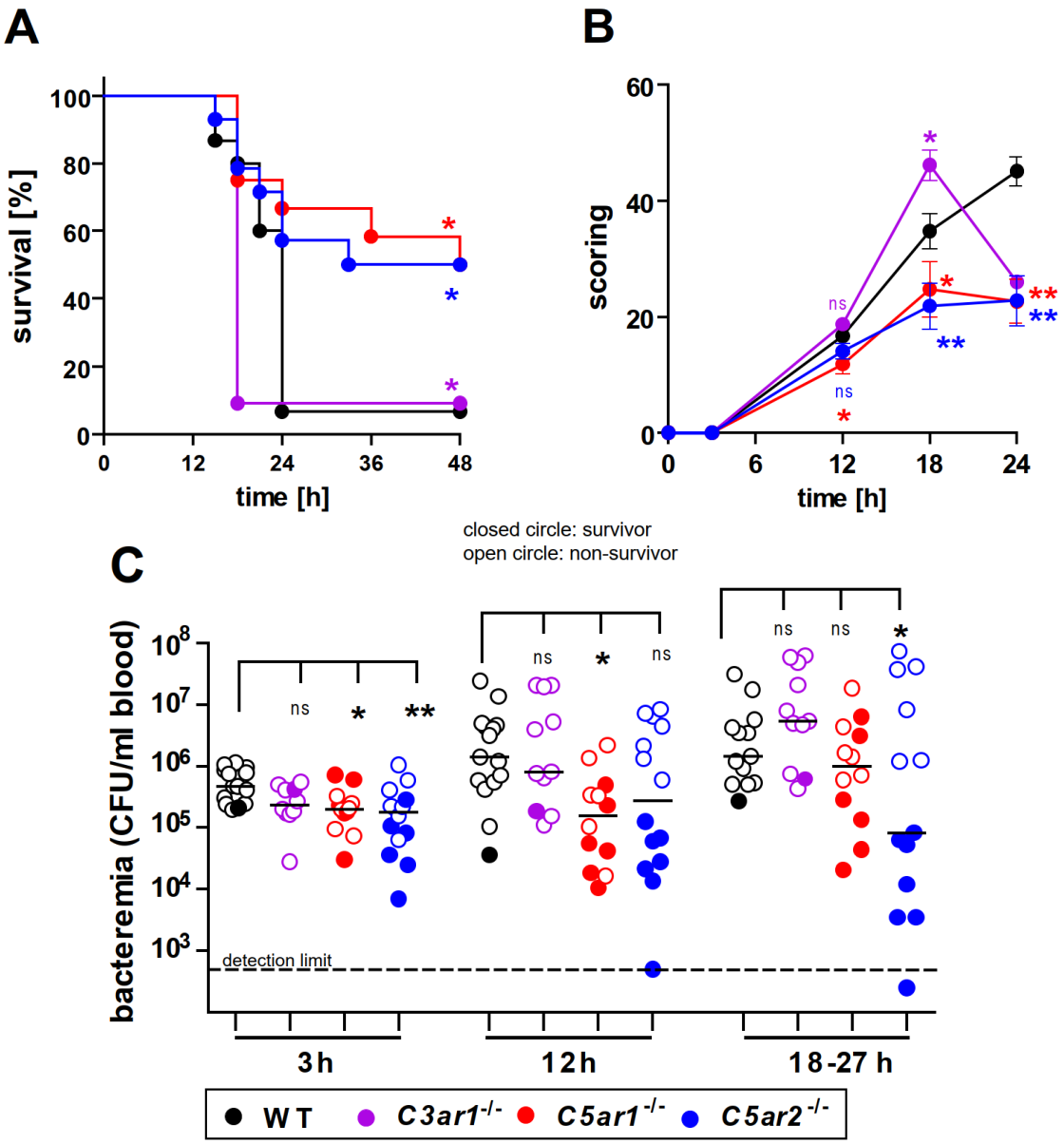

Figure 3: Different outcome in ATRs knock-out mice in experimental induced sepsis. ATR knock-out mice were injected i.p. with 10^5 CFU of Mc58 per animal, together with a dose of iron dextran at 0 h. An additional bolus of iron dextran was given 12 h post infection. All mice were closely monitored for 48 h and euthanized upon reaching the humane endpoint. Blood samples were obtained 3 h and then in 12 h intervals post infection or upon reaching the humane endpoint, for assessing the bacterial burden of each mouse. **A** shows the survival rates of the different genotypes upon infection. **B** displays the scoring points given to mice. **C** presents the bacterial burden in the different knock-out mice. Open circles represent mice that reached the humane endpoint. Closed circles stand for mice that did not reach the humane endpoint during the course of the experiment. 500 CFU/ml was the limit of detection for the bacterial burden. ns, not significant; *, P<0,05; **, P<0,01; in Mantel-Cox test for the survival curves compared to WT mice; in one-way ANOVA with Bonferroni's *post hoc* test for the bacterial burden and in Kruskal-Wallis with Dunnett's *post hoc* test for the clinical score. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

In strong contrast to those findings, *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice showed a significantly increased survival rate, which was as high as 50%. Consistent with that, both knock-out mice strains also showed a significantly reduced *Nme* level as early as three hours post infection. During the course of the experiment both knock-out mice strains showed a trend for a reduced bacterial burden, especially in mice which survived the sepsis (closed circles). The bacterial counts were significantly reduced 3 h and 12 h post infection for *C5ar1*$^{-/-}$ mice and 3 h and 18-27 h post infection for *C5ar2*$^{-/-}$ mice (Fig. 3C). In line with that, also their clinical scores were significantly reduced for 18 h and 24 h post infection and for *C5ar1*$^{-/-}$ mice also already 12 h post infection (Fig. 3B). With these findings it could be shown that the expression of C3aR is very important for the survival of mice in meningococcal sepsis. Contrary to that finding, it was very interestingly that not only the lack of C5aR1, in accordance to Herrmann *et al.* (93), but also the lack of C5aR2 was beneficial for the survival of meningococcal sepsis in mice.

3.2.2. C3aR expression is also important in IMD with a nonlethal *Nme* dose

Comparing the outcome of meningococcal sepsis in ATR knock-out mice revealed a higher survival rate in mice lacking either C5aR1 or C5aR2, compared to the WT mice, but a surprisingly worsened outcome in mice lacking C3aR1, which reached the humane endpoint even earlier than the WT mice. Seeing this detrimental effect, cohorts of WT and *C3ar1*$^{-/-}$ mice were injected i.p. with a nonlethal dose of $5x10^4$ CFU of *Nme* per animal (Fig. 4). Using this nonlethal dose, all WT mice survived the infection, whereas still 40% of the *C3ar1*$^{-/-}$ mice succumbed to the infection (Fig. 4A). In line with that, the clinical score was increased significantly 12 h post infection and also at a later time point of 24 h post infection (Fig. 4B). The clinical score was accompanied by a significantly increased bacterial burden 12 h post infection of the *C3ar1*$^{-/-}$ mice, when compared to the WT mice. These results highlight the findings from Fig. 3, which showed a severe outcome in *C3ar1*$^{-/-}$ mice, as the lack of C3aR had already a significant impact on the overall scoring and bacterial burden of these mice with an injection of a nonlethal dose of *Nme*. Therefore, C3aR seems to be of utmost importance for fighting off meningococcal infections in this meningococcal sepsis in a murine setting.

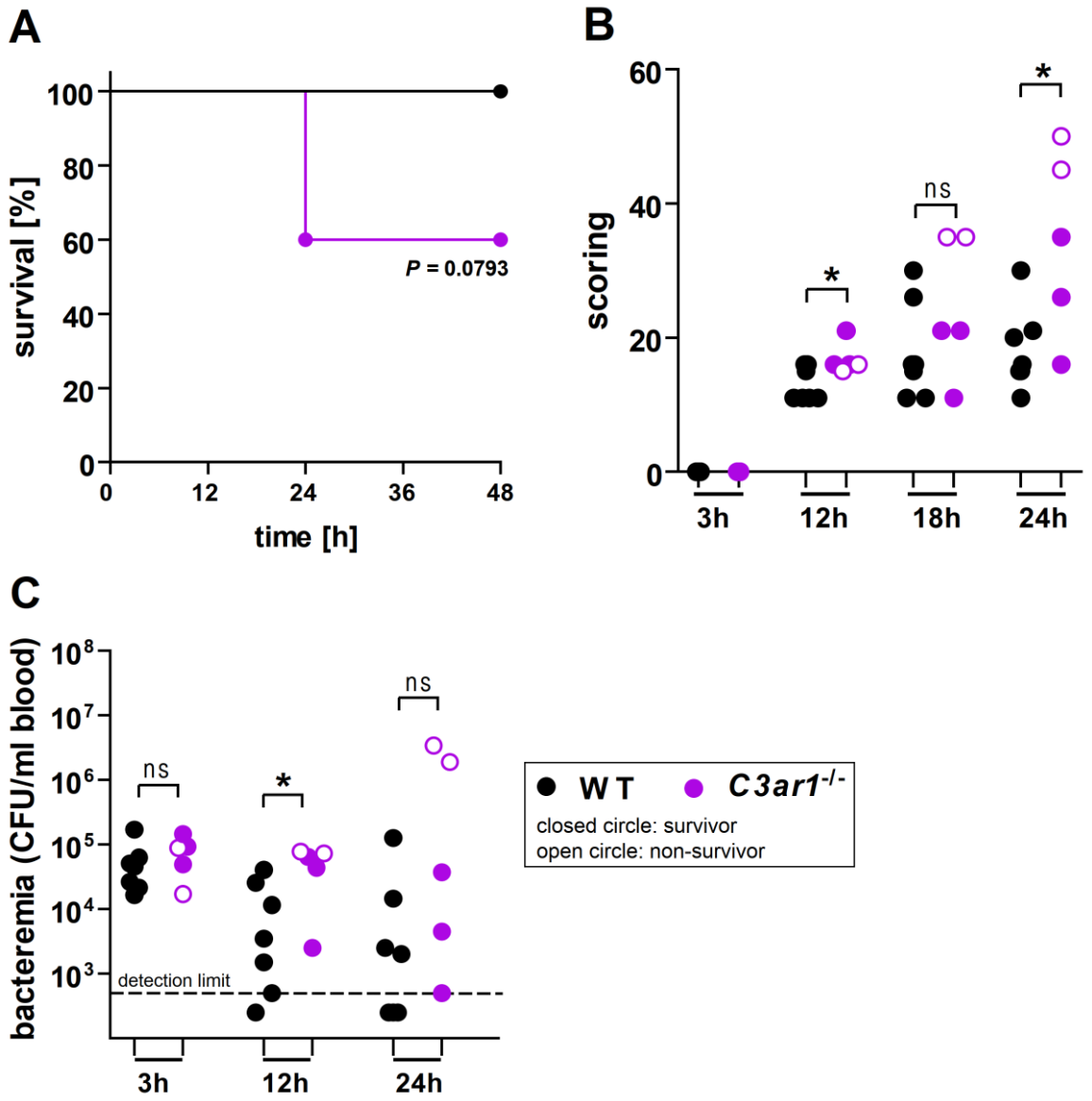

Figure 4: Lack of C3aR worsen outcome of IMD in nonlethal setting.
WT and C3aR knock-out mice were injected i.p. with $5x10^4$ CFU of Mc58 per animal, together with a dose of iron dextran at 0 h. An additional bolus of iron dextran was given 12 h post infection. All mice were closely monitored for 48 h and euthanized upon reaching the humane endpoint. Blood samples were obtained 3 h and then in 12 h intervals post infection or upon reaching the humane endpoint, for assessing the bacterial burden of each mouse. **A** shows the survival rates of the different strains. **B** presents the scoring points given to each mouse at the indicated time points. **C** displays the bacterial burden in the different knock-out mice at the given time points. 500 CFU/ml was the limit of detection. ns, not significant; *, $P<0,05$; in Mantel-Cox test for the survival curves; in Mann-Whitney test for the clinical score and bacteremia. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

3.2.3. Reduced cytokine responses in all three ATRs knock-out mice in IMD

Comparing the outcome of meningococcal sepsis in anaphylatoxin receptor knock-out mice, revealed a high impact of those on the outcome of IMD (Fig. 3A). ATRs are involved in many different cellular processes and also in the production of cytokines (74). To gain deeper insight into this relation in IMD and to understand further possible underling mechanisms, tail vein blood was drawn 12 h post infection from ATR knock-out mice. The cytokine profile of those plasma samples was then analyzed via ELISA and LEGENDplex™ (Fig. 5). As expected, all four different mouse strains showed a similar low background level for the tested cytokines in uninfected conditions. A significantly reduced cytokine level was measured in all three ATR knock-out mice, compared to WT mice, for the pro-inflammatory cytokines CXCL-1 (70) and IL-6 (40). Surprisingly, *C3ar1*$^{-/-}$ mice showed like *C5ar1*$^{-/-}$ mice a trend for an overall reduced cytokine profile, however only *C5ar1*$^{-/-}$ mice showed significantly reduced levels for TNF-α, IFN-γ and MCP-1 in addition to CXCL-1 and IL-6. In contrast to that, the other cytokine levels measured in *C5ar2*$^{-/-}$ mice were on a similar level as in WT mice. These data show that the absence of each ATR, even the C3aR, is leading to a reduced secretion of the pro-inflammatory chemokine/cytokine CXCL-1 and IL-6. In fact, tail vein blood measurements by ELISA showed that the secretion of CXCL-1 was already significantly reduced as early as 3 h post infection (Fig. 6). Taken together, a reduced cytokine profile might be beneficial, as *C5ar1*$^{-/-}$ mice showed several significantly reduced cytokine levels, but significantly reduced cytokine levels were also observed in *C5ar2*$^{-/-}$ and even in *C3ar*$^{-/-}$ mice. This shows that the reduced cytokine and chemokine response in ATR knock-out mice might not be solely responsible for the detrimental outcome of IMD seen in *C3ar*$^{-/-}$ and WT mice, versus the beneficial outcome seen in *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice (Fig. 3A).

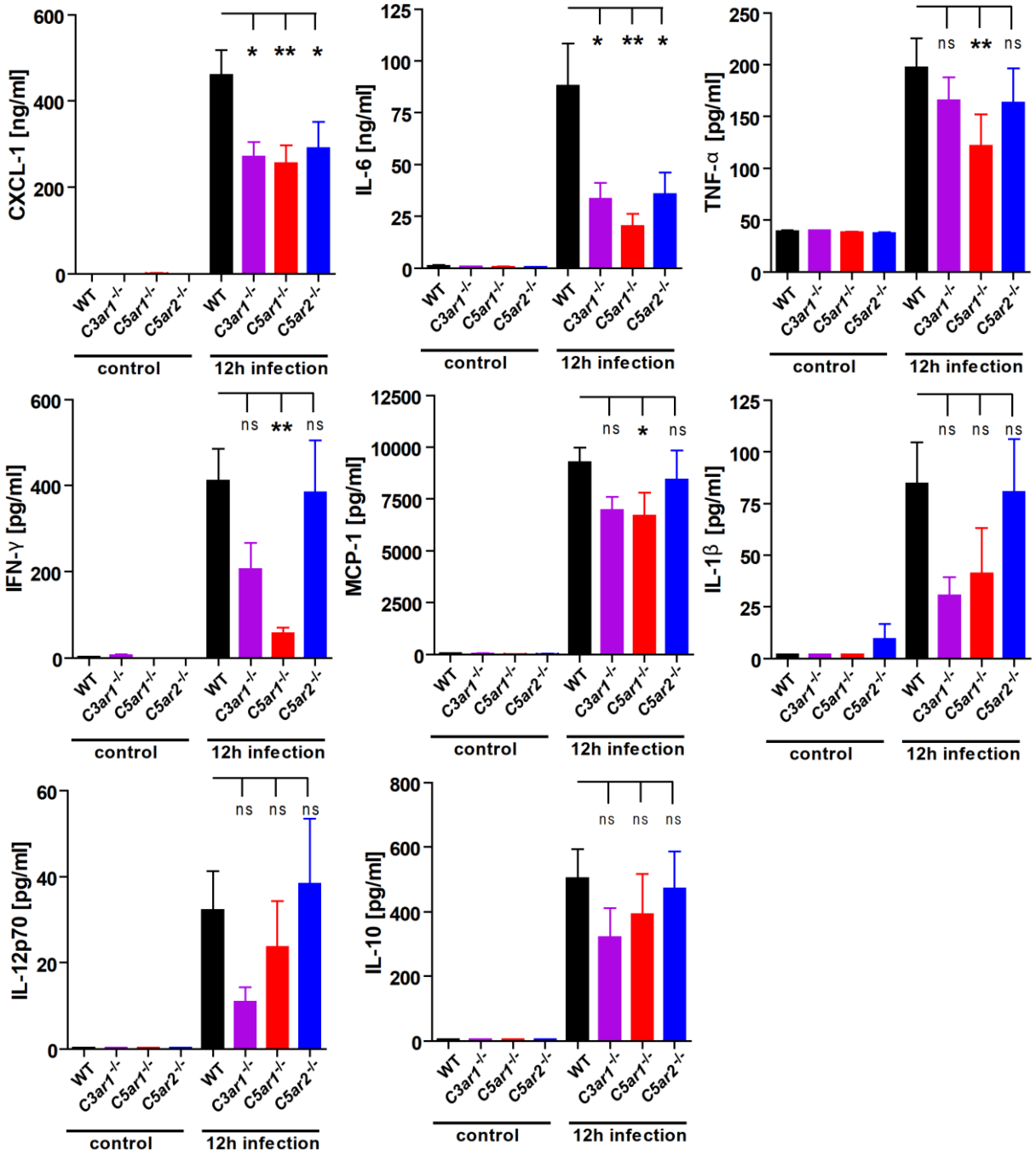

Figure 5: Cytokine profile of ATR knock-out mice 12 h post infection
Mice were injected i.p. with 10^5 CFU of MC58 per animal, as displayed in Fig. 3. Tail vein blood was collected before and 12 h post infection from those mice. The plasma samples were measured for the cytokine secretion. Standard deviation and means are plotted. ns, not significant; *, P<0,05; **, P<0,01; in one-way ANOVA using Dunnett's multiple *post hoc* test with the WT samples as a reference. The data were published in Muenstermann *et al*., 2019, Virulence (157).

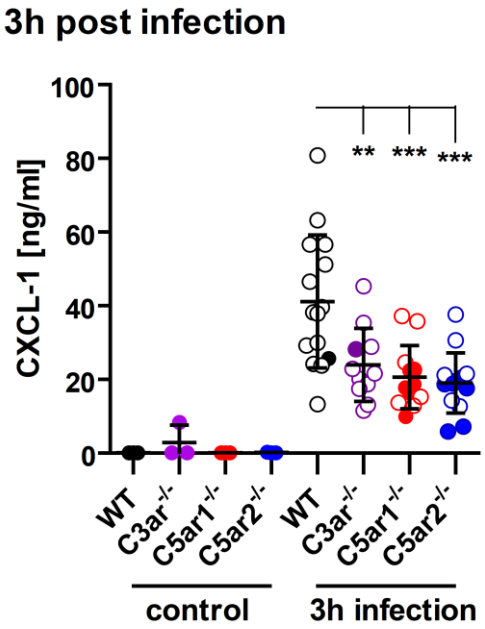

Figure 6: CXCL-1 measurement in ATR knock-out mice 3 h post infection
Mice were injected i.p. with 10^5 CFU of MC58 per animal, as displayed in Fig. 3. Tail vein blood was collected from those mice 3 h post infection. The plasma samples were measured for CXCL-1 secretion. Open circles represent mice that reached the humane endpoint. Closed circles symbolize mice that did not reach the humane endpoint during the experiment. Standard deviation and mean are presented with lines. **, P<0,01; ***, P<0,005; in one-way ANOVA using Dunnett's multiple *post hoc* test with the WT samples as a reference. The data were published in Muenstermann *et al*., 2019, Virulence (157).

3.3. *In vivo* – pharmacologic targeting of ATRs

3.3.1. Therapeutically targeting C5aR1 and C5aR2 *in vivo* ameliorated meningococcal sepsis

Infection of ATR knock-out mice with a lethal dose of *Nme* revealed that mice lacking C5aR1 or C5aR2 showed an ameliorated disease progression. This stood in strong contrast to mice lacking the C3aR, which reached the humane endpoint even faster than WT mice upon infection via *Nme*. Discovery of these interesting findings lead to the question, whether this effect could be complemented by therapeutically targeting the three ATRs. Blocking or activation of ATRs can be accomplished with different compounds. The C3aR can be blocked using a chemical compound named SB290157 (143). As signaling via C3aR seems to be important for the disease outcome *in vivo*, it stands to reason that activation of C3aR might increase the survival rate. Therefore, the superpotent peptide (WWGKKYRASKLGLAR), designed by Ember *et al.*, which strongly activates C3aR was used (144). For blocking C5aR1 the circular PMX205 was used (145, 146). PMX205 already showed a great potential in meningococcal sepsis as shown by Herrmann *et al.* (93). Even though extensively searched for, there is still no reagent specifically blocking C5aR2 in mice (84). Yet, there is the peptide $A8^{\Delta 71\text{-}73}$ that blocks C5aR2 as well as C5aR1 simultaneously (147). In this work, $A8^{\Delta 71\text{-}73}$ was therefore used to block C5aR2 together with C5aR1 in mice. Cohorts of mice were injected i.p. with the different reagents that interfere with ATRs and infected with a lethal dose of *Nme* (Fig. 7A). The control group of WT mice, injected only with the vehicle, showed that under normal circumstances the administered dose of *Nme* is lethal, as almost all mice reached the humane endpoint. In contrast to that, as it has been shown previously by Herrmann *et al.*, PMX205, blocking the C5aR1, increased the survival rate of the WT mice significantly. A similar effect could be seen in WT mice treated with $A8^{\Delta 71\text{-}73}$, where C5aR2 and C5aR1 were blocked simultaneously. The effect when both C5aRs were blocked was slightly lower, compared to blocking C5aR1 alone with PMX205. This could be explained by the linear peptide $A8^{\Delta 71\text{-}73}$ binding differently to the C5aRs, compared to the circular PMX205 (145-147). In contrast to that, neither blocking C3aR with SB290156 nor activating it with the superagonist changed the survival rate of the WT mice significantly. The compound SB290157 has also been shown to have agonistic capabilities (158) and the superagonist might be quickly degraded *in vivo* as it is a linear peptide, which could explain the absent changes in the survival rates.

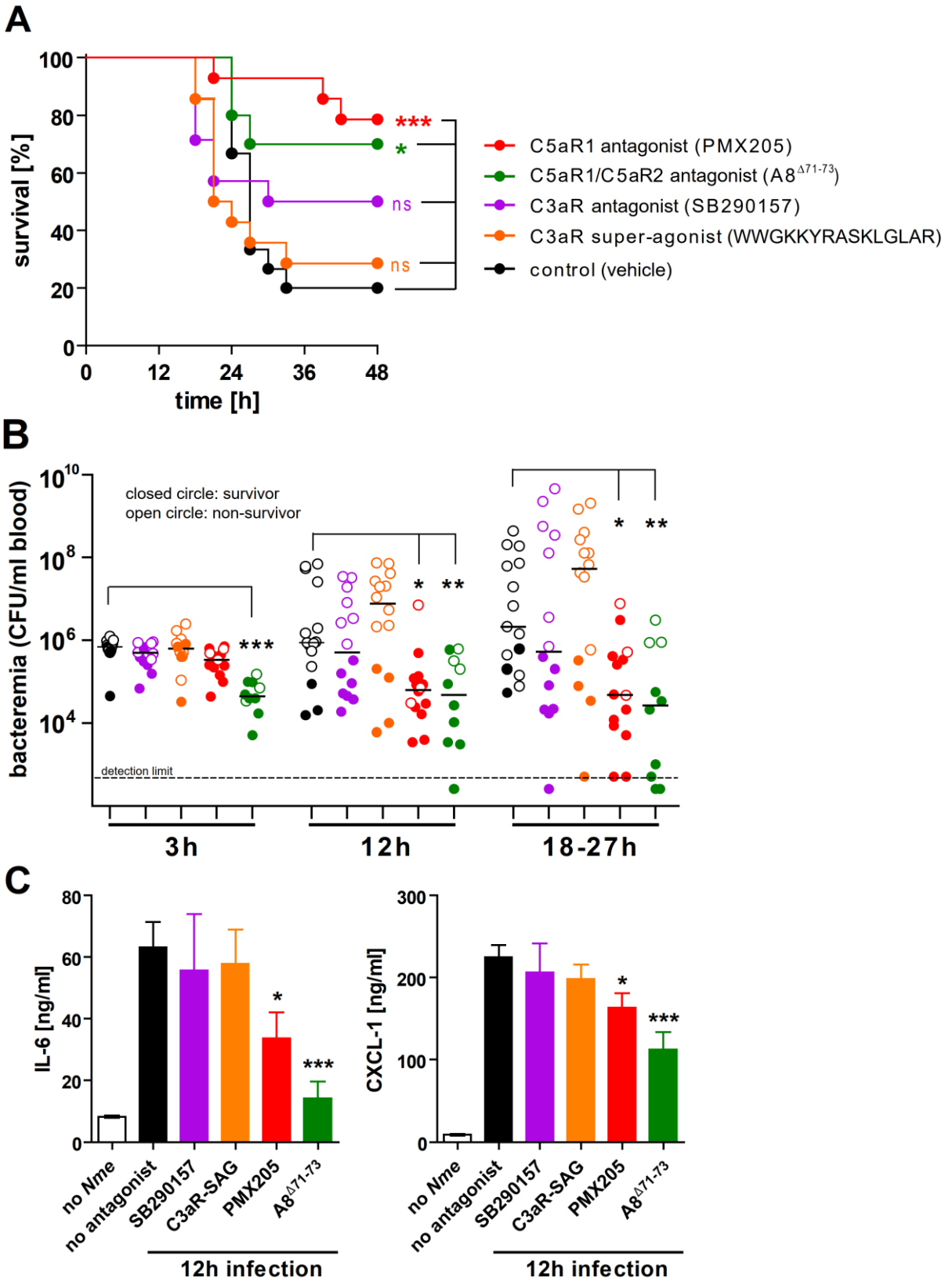

Figure 7: Increased survival rates in PMX205 and A8$^{\Delta 71-73}$ treated mice.
WT mice cohorts were injected i.p. starting before and during the infection with sterile 5% glucose solution alone, with a superagonist (WWGKKYRASKLGLAR) for C3aR, with the C3aR antagonist SB290157, with A8$^{\Delta 71-73}$ blocking C5aR1 and C5aR2 or with PMX205 blocking C5aR1 alone. Infection was done i.p. with 10^5 CFU of Mc58 per animal, together with

a dose of iron dextran at 0 h. An additional bolus of iron dextran was given 12 h post infection. All mice were closely monitored for 48 h and euthanized upon reaching the humane endpoint. Blood samples were obtained 3 h and then in 12 h intervals post infection or upon reaching the humane endpoint, for assessing the bacterial burden of each mouse. **A** shows the survival rates of the different treatments. **B** displays the bacterial burden in the differently treated mice. **C** shows ELISA measurements of IL-6 and CXCL-1 plasma levels in mice 12 h post infection. 500 CFU/ml was the limit of detection for the bacterial burden. ns, not significant; *, $P<0,05$; **, $P<0,01$; ***, $P<0,001$; in Mantel-Cox test for the survival curves compared to WT mice; in one-way ANOVA with Bonferroni's *post hoc* test for the bacterial burden and in Kruskal-Wallis with Dunnett's *post hoc* test for the clinical score. The data were published in Muenstermann *et al*.; 2019; Virulence (157).

The bacterial burden (Fig. 7B) in the control group, the SBS90157 and the superagonist treated group mirrors the low survival rates and shows no changes in the number of bacteria in these groups. Contrary to that, the bacterial burden in mice treated with PMX205 or $A8^{\Delta 71\text{-}73}$ was significantly reduced 12 h and 18-27 h post infection compared to the control group. In the case of the $A8^{\Delta 71\text{-}73}$ treatment, the number of bacteria was already significantly reduced 3 h post infection. To further look into the infection responses and possible differences, cytokine tests via ELISA for the proinflammatory IL-6 and the neutrophil chemoattractant CXCL-1 were performed (Fig. 7C). These tests revealed again no changes among the control group and the groups treated with SB290157 or the superagonist. Significantly reduced levels were detected in mice treated with PMX205, for C5aR1 blockade, or mice treated with $A8^{\Delta 71\text{-}73}$, for C5aR1 and C5aR2 blockade. All together, these experiments showed that the positive effect seen in lethally infected *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice, could be replicated targeting these receptors with PMX205 or $A8^{\Delta 71\text{-}73}$ in WT mice. The results seen in *C3ar*$^{-/-}$ mice were not replicated with the used antagonist and also an activation of that receptor did not alter the survival significantly. These results are showing a difference in *Nme* infection clearance dependent on C5aR1 and C5aR2 and a changed immunological reaction, displayed by a reduced level of IL-6 and a reduced level of the neutrophil chemoattractant CXCL-1, without C5aR1 and C5aR2 signaling.

3.4. *Ex vivo* analysis - mouse whole blood

3.4.1. Oxidative burst and degranulation reduced in PMNs of *C5ar1*$^{-/-}$ mice, but not in PMNs of *C3ar1*$^{-/-}$ - or *C5ar2*$^{-/-}$ mice

The depletion experiments revealed that neutrophils are important in clearing meningococcal infections and reduced levels of the neutrophil chemoattractant CXCL-1 were measured without ATR expression *in vivo*. Therefore, neutrophils are analyzed further in this part. Neutrophils are characterized as first responders in bacterial infections. They are capable of killing bacteria using several different mechanisms, can stimulate the release of cytokines to attract further neutrophils and are recruited quickly in great numbers to fight off infections (70). As neutrophils are important players in meningococcal sepsis, *ex vivo* studies were conducted to analyze whether their reactions to *Nme* might be changed in the absence of one of the three ATRs. To be able to mimic the normal blood composition the best and without eliminating the complement system, which is important for phagocytosis or the oxidative burst for example in humans (159), hirudin was used in the whole blood infection assays as an anti-coagulant. Hirudin is a thrombin-specific inhibitor and does not interfere with the complement system in contrast to heparin for example (160). Measuring the phagocytosis capability using GFP positive *Nme*, no differences between the different ATR knock-out PMNs was seen (Fig. 8C). Another important mechanism of neutrophils is the oxidative burst (Fig. 8A). DHR123 oxidizes in the presence of reactive oxygen intermediates to the green fluorescent rhodamine-123, which can then be measured via flow cytometry analyses (149). The PMA positive control showed the same level of oxidative burst in all samples, indicating that neutrophils lacking one of the receptors are still able to do an oxidative burst, when stimulated with PMA. Looking at *Nme* as a stimulus alone, neutrophils lacking C5aR1 mounted a weaker oxidative burst compared to WT neutrophils, in line with previous finding published by Herrmann *et al.* (93). In contrast to that, neutrophils lacking C3aR or C5aR2 did not show a reduction in the oxidative burst compared to the WT mice. Similar results were obtained, when measuring degranulation by FACS staining for CD11b surface abundance (Fig. 8B). When PMNs were infected with *Nme*, the measured CD11b surface expression was lower in cells harvested from *C5ar1*$^{-/-}$ mice, compared to cells from WT mice, but changes were seen in PMNs harvested from *C3ar*$^{-/-}$ or *C5ar2*$^{-/-}$ mice. The positive control with PMA stimulation showed no difference regarding the degranulation capability in all four groups. However, a reduced level of CD11b expression in *C5ar1*$^{-/-}$ PMNs was already visible in the PBS treated negative control samples. This could suggest a lower initial abundance of CD11b on the surface of C5aR1 lacking neutrophils.

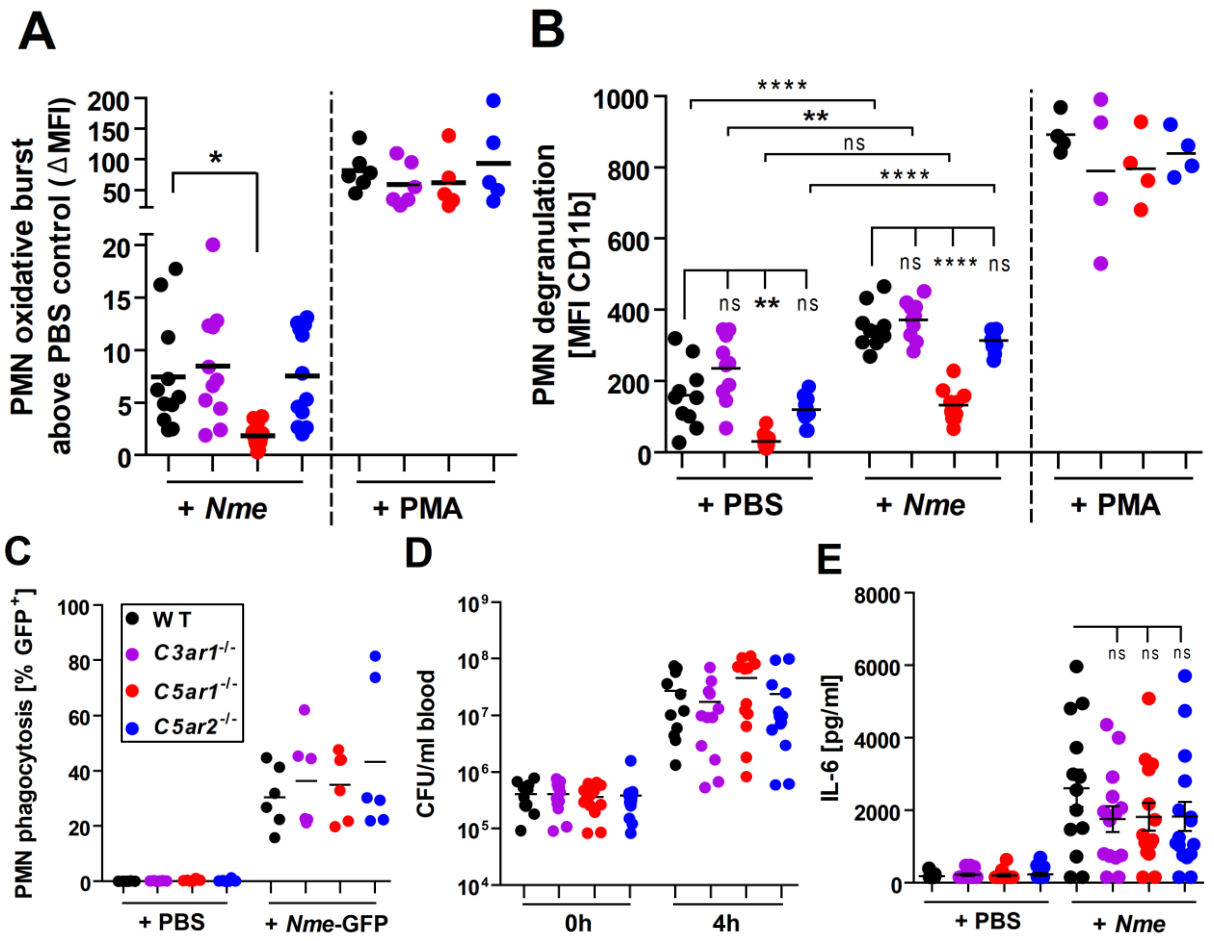

Figure 8: No differences in functions of PMNs from *C3ar*$^{-/-}$, *C5ar1*$^{-/-}$ or *C5ar2*$^{-/-}$ mice, except for degranulation and oxidative burst in *C5ar1*$^{-/-}$ mice, stimulated with *Nme*.
Whole blood was collected from either WT, *C3ar*$^{-/-}$, *C5ar1*$^{-/-}$ or *C5ar2*$^{-/-}$ mice. Those whole blood samples were infected with a final concentration of 10^8 CFU/ml of *Nme*-GFP **(A)**, 10^7 CFU/ml of *Nme* **(B)** and **(C)** or with 10^6 CFU/ml in **(D)**. PBS, PMA or isotype-FITC antibodies were used as controls. Neutrophils were labeled with anti-Ly6G-APC antibodies (**A**, **B** and **C**). Oxidative burst was measured with DHR123 staining (**A**). Degranulation was measured by staining with anti-CD11b-FITC (**B**). The samples were analyzed with a BD FACSCanto. Phagocytosis **(A)** is displayed as percentage of GFP$^+$/Ly6G$^+$ cells. The oxidative burst **(B)** is displayed as the MFI of FL-1$^+$ cells, with the signal from the PBS control being subtracted to eliminate the slight autofluorescence of *C5ar2*$^{-/-}$ mice due to GPF expression. Degranulation **(C)** is displayed as MFI of FITC$^+$ cells, with the isotype-FITC labeled antibody signal subtracted. **(D)** Bacterial count 4 h post infection is displayed as CFU/ml. **(E)** IL-6 secretion was measured via ELISA in plasma samples taken 4 h post infection, with or without bacteria. *, P<0,05; **, P<0,01; ***, P<0,001; in one-way ANOVA with Bonferroni's *post hoc* test. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

As a fourth test a possible difference in bacterial killing capacities was assessed (Fig. 8D). Therefore, the growth of bacteria in whole blood after 4 h was compared among the groups. Even though the spread of colonies counted 4 h post infection was quite high, the overall picture showed no differences among the samples from the different ATR knock-out groups. In addition, IL-6 measurement in the samples also did not show significant changes (Fig. 8E). The importance of the usage of a whole blood system with an intact complement system is displayed

in Fig. 9. The phagocytosis of PMNs was significantly reduced in mice lacking C3, showing that this process is greatly depend on this complement factor. All in all, these analyses showed that the lack of C5aR1 on PMNs, directly has an impact on their oxidative burst and degranulation. In contrast to that, the cellular responses of PMNs did not seem to be impaired by the lack of either C3aR1 or C5aR2. Furthermore, phagocytosis by PMNs is greatly dependent on C3 expression.

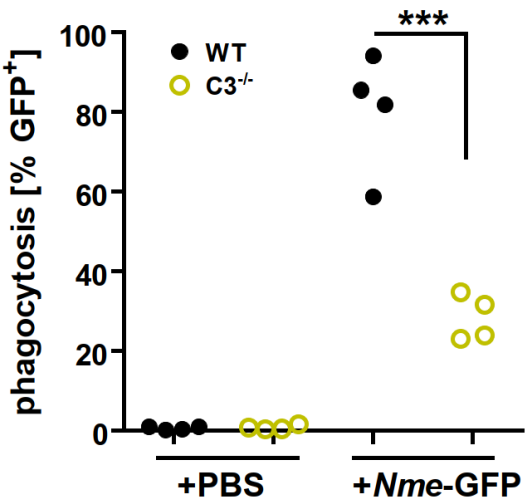

Figure 9: Reduced phagocytosis of *Nme* in C3 deficient mice.
Whole blood was taken from either WT or *C3*$^{-/-}$ mice and infected with a final concentration of 10^8 CFU/ml of *Nme*-GFP. Neutrophils were marked with anti-Ly6G-APC antibodies and the samples were analyzed via flow cytometry. Phagocytosis is displayed as percentage of GFP$^+$/Ly6G$^+$ cells. ***, *P*<0,001; in two-tailed Student's T-test. The data were published in Muenstermann *et al*.; 2019; Virulence (157).

3.5. *Ex vivo* analysis - human whole blood

3.5.1. ATR differently affect oxidative burst, degranulation and phagocytosis of human whole blood PMNs in an *ex vivo Nme* infection model

Nme is primarily an asymptomatic colonizer of the nasopharyngeal tract and can only be found in humans (26). For studying the direct influences of ATRs on a fulminant sepsis, an established intraperitoneal animal infection model was used (137). Nevertheless, in order to take the natural human specificity into account and to mimic a human meningococcal infection, a human whole blood system was chosen for the following experiments. In the mouse model, the lack of ATRs had a detrimental outcome on the sepsis and to test for similar effects in a human setting, different reagents were used targeting the complement system and the ATRs. The human whole blood infections were analyzed for the same parameters, oxidative burst, degranulation, phagocytosis and cytokine release, as in the *ex vivo* murine whole blood experiments. As a control, the whole complement cascade was blocked using the peptide Cp20, named compstatin. It binds to C3 and blocks its interaction with the C3-convertases, which leads to a stop of the downstream cascade and ultimately prevents the release of C3a and C5a (Fig. 11) (161). The ATR C3aR was blocked using the selective nonpeptidic antagonist SB290157 (143). Stimulation of the same receptor was done using the superagonistic linear peptide C3aR-SAG (144). The ATR C5aR1 was specifically blocked with the circular peptide PMX53 (145, 162) and C5aR2 was blocked using anti-C5aR2-antibodies from clone 1D9-M12 (163). Additionally, the antagonist $A8^{\Delta 71\text{-}73}$ blocking C5aR1 and C5aR2 simultaneously (147) was used in these assays. In figure 1 and published in Herrmann *et al.* in 2018 (93), it was shown that next to the complement also cellular involvement is important for a positive outcome of IMD. One of those important first responder cell types are phagocytic neutrophils, which were specifically analyzed in a human whole blood system. Hirudin anticoagulated human whole blood was drawn and treated with one of the different antagonists or agonists described above. These samples were then infected with Mc58, for assessing the oxidative burst or the degranulation, or with MC58Δ*csb*-GFP to analyze possible changes in the phagocytic capabilities. The results are displayed in Fig. 10. The results for the oxidative burst analysis are displayed in Fig. 10A. The control samples of human whole blood being infected only with *Nme* showed significant increase of the oxidative burst by PMNs. Using compstatin to block the whole complement cascade resulted in a significant oxidative burst reduction, indicating that this process is strongly dependent on complement mediators.

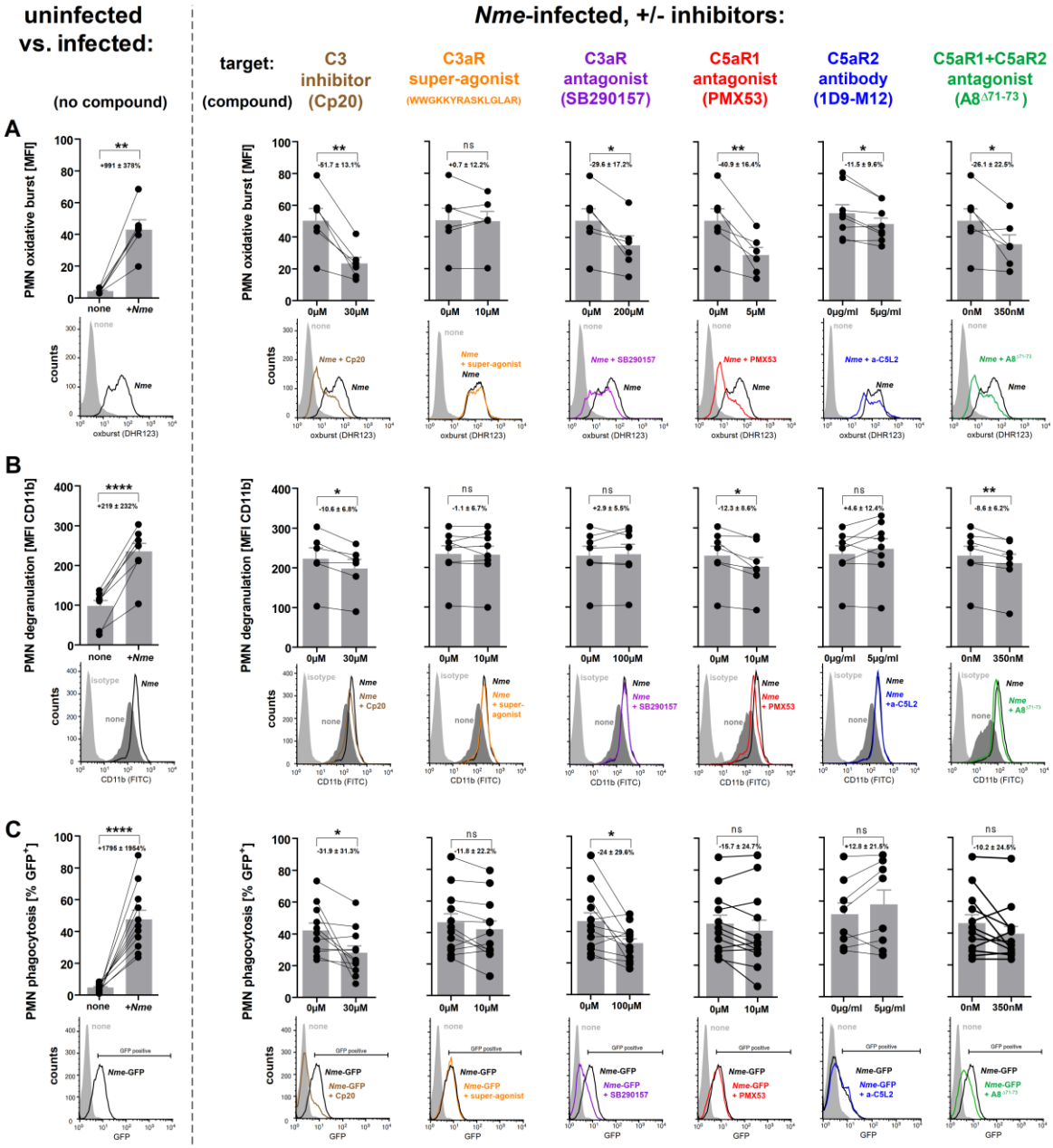

Figure 10: Blockade or stimulation of ATRs alters phagocytosis, oxidative burst and degranulation in a human whole blood infection model

Human whole blood was drawn and co-incubated with either compstatin to block the complement cascade, with C3aR-SAG to activate the C3aR, with SB290157 to block the C3aR, with PMX53 to activate the C5aR1, with anti-C5aR2 antibodies to block C5aR2 or with $A8^{\Delta 71-73}$ to block C5aR1 and C5aR2. The whole blood samples were treated with RPMI as a control or infected with a final concentration of either 10^7 CFU/ml of *Nme* (**A** and **B**) or 10^8 CFU/ml of *Nme*-GFP (**C**). The samples for the oxidative burst measurements (**A**) were stained with DHR123. The samples for the degranulation assay (**B**) were stained with either anti-CD11b-FITC or isotype-FITC-labeled antibodies. All samples were fixated in 4% PFA for flow cytometry analysis. Human PMNs were gated for their specific size and granularity. The oxidative burst (**A**) is displayed as the MFI of FL-1^+ PMNs. Degranulation (**B**) is displayed as the MFI of $FITC^+$ PMNs. Phagocytosis (**C**) is displayed as percentage of GFP^+ PMNs. The measurements for the donors are displayed with connected dots in the graphs. The bars

represent the mean ± standard error of the mean. The values on top of the graphs display the relative reduction compared to the measurements without reagents in percentage ± standard deviation. Each gating is shown below the graph in a representative histogram. ns, not significant; *, P<0,05; **, P<0,01; ****, P<0,0001; in two-tailed Student's T-test. The data were published in Muenstermann *et al*.; 2019; Virulence (157).

The blockade of C3aR, C5aR1, C5aR2 or C5aR1 and C5aR2 simultaneously, showed a significant reduction of the oxidative burst, revealing that the anaphylatoxins released in the complement cascade must play an important role in the oxidative burst by human whole blood PMNs, which can be reduced by blocking either one of the three ATRs. In contrast to that, the activation of the C3aR via the superagonist, did not show a significant increase or decrease in the oxidative burst. The degranulation, measured by the surface expression of CD11b, is displayed in Fig. 10B. Upon *Nme* stimulation of the human whole blood, a strong degranulation in the PMNs could be observed in the control samples. This reaction also depends to some extend on the complement system and its mediators, as compstatin also significantly reduced the degranulation. A significant reduction in degranulation, could only be observed when the C5aR1 was blocked, either alone with PMX53 or in combination with the C5aR2 by using $A8^{\Delta 71\text{-}73}$. As there was no significant reduction in the samples, where C5aR2 was blocked alone by the antibody, the effect of $A8^{\Delta 71\text{-}73}$ is most likely mediated through the blockade of C5aR1. Activation of the C3aR with the superagonist, also did not affect degranulation significantly. Phagocytosis was measured, using GFP expressing *Nme* and the results are displayed in Fig. 10C. Upon infection with *Nme*, a significantly strong increase of *Nme* uptake can be observed by PMNs in the untreated control samples. This process is also dependent to some extent on the complement system, as blocking the cascades with compstatin also here significantly reduced the uptake of *Nme*. Surprisingly, only the blockade of C3aR was followed by a significant reduction of the phagocytosis. A trend could be observed for C5aR1 blockaded for some human whole blood samples, however, this trend was not significant. The activation of the C3aR with the superagonist did not change the phagocytosis. In line with the *ex vivo* PMN experiments in mice, only the blockade of C5aR1 showed a significant reduction in degranulation of human whole blood PMNs. In contrast to that, the oxidative burst was decreased in human whole blood upon blocking either of the three ATRs. Surprisingly, blocking C3aR in human whole blood PMNs revealed a decrease of phagocytosis capacity, which was not seen when any of the other ATRs was blocked. Stimulation of C3aR with the superagonist, did not affect any of the three measured cellular responses mediated by human whole blood PMNs. Additionally, experiments with compstatin, blocking the whole complement cascade, revealed a strong dependence of the different cellular responses on the complement system with its mediators.

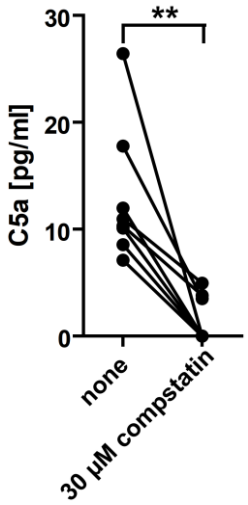

Figure 11: Compstatin efficiently blocks C5a release upon *Nme* stimulation
Human whole blood was infected with a final concentration of 10^6 CFU/ml of *Nme* and co-incubated with or without compstatin. 20 mM EDTA was added 30 min post infection and the C5a concentration was measured with ELISA. **, $P<0,01$; in two-tailed Student's T-test. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

3.5.2. ATR strongly affect cytokine release of human whole blood PMNs in an *ex vivo Nme* infection model

In an infection, neutrophils are recruited in vast numbers to infection sides and in this mobilization process the chemoattractant IL-8 plays an important role (70). Additionally, IL-8 is also an activator of neutrophils and thereby involved in the oxidative burst and degranulation of those cells (164). To analyze possible involvement of the anaphylatoxins in IL-8 secretions in meningococcal infections, human whole blood samples were infected with *Nme* and measured for the amount of secreted IL-8 by ELISA. The results are displayed in Fig. 12. Upon *Nme* stimulation the amount of IL-8 rose significantly in the human whole blood control samples. The control group with compstatin showed that this reaction is to some extend dependent on the complement system and its mediators, as the secretion of IL-8 was reduced without complement activation. The whole measurement showed a strong interplay between the complement system, the anaphylatoxin receptors and the secretion of IL-8. Blockade of all three anaphylatoxin receptors C3aR, C5aR1 and C5aR2 led to a significantly reduced amount of IL-8 secretion.

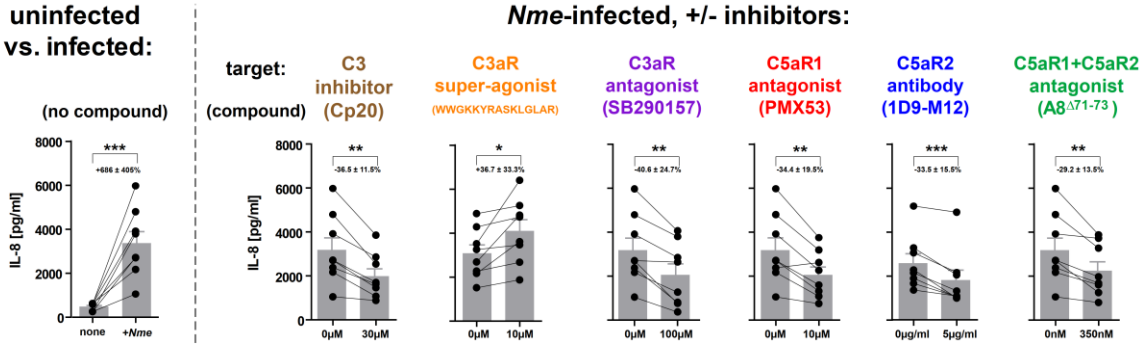

Figure 12: Differential IL-8 secretion after blocking or stimulating ATRs in human whole blood

Human whole blood was drawn and co-incubated with either compstatin to block the complement cascade, with C3aR-SAG to activate the C3aR, with SB290157 to block the C3aR, with PMX53 to activate the C5aR1, with anti-C5aR2 antibodies to block C5aR2 or with $A8^{\Delta 71-73}$ to block C5aR1 and C5aR2. The whole blood samples were then treated with RPMI as a control or infected with a final concentration of 10^6 CFU/ml of *Nme*. All samples were incubated for 90 min, rotating at 37 °C. IL-8 secretion was measured with ELISA. *, $P<0,05$; **, $P<0,01$; ***, $P<0,001$; in two-tailed Student's T-test. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

Interestingly, the C3aR-SAG had no impact on the cellular processes, the oxidative burst, the degranulation or the phagocytosis of neutrophils, but the human whole blood stimulation via the C3aR-SAG yielded a significant increase in IL-8 secretion. These experiments show that in the clearance of *Nme* infections, cellular processes and mobilizations must be important factors, next to the undoubtably important direct complement mediated killing of *Nme*. Furthermore, these experiments show that these processes are directly intertwined with the complement activation, the release of anaphylatoxins in the process and the subsequent activation of ATRs on different cell types, which leads to an important immune reaction in *Nme* clearance.

To rule out possible effects of the used compounds on *Nme*, which could have influenced the results, human whole blood was co-incubated with the used compounds in different concentrations and infected with bacteria. As figure 13 displays, none of the compounds in the concentration used in the experiments in figure 10 and figure 12 or in higher or lower concentrations were negatively affecting the viability of *Nme*. The number of bacteria varies between the different donors, as the whole blood of each human responds differently to the *Nme* infection.

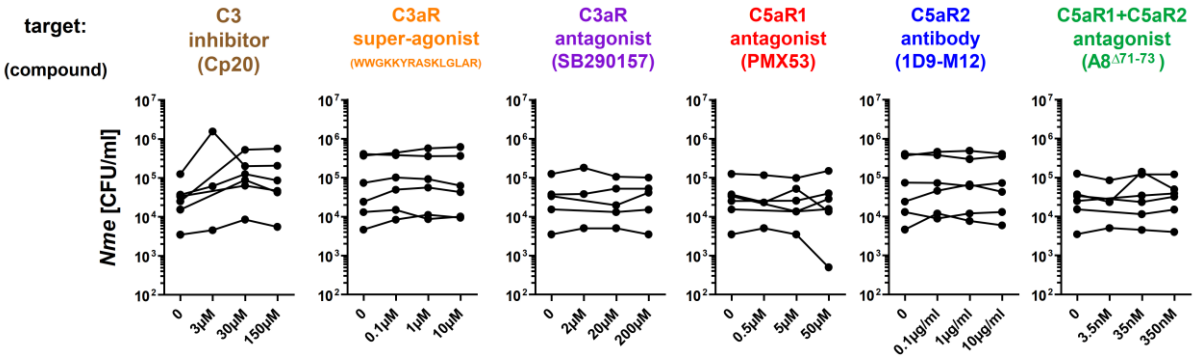

Figure 13: *Nme* viability was not impaired by compounds used to target the ATRs or C3
Human whole blood was drawn and co-incubated for 10 min at RT with RPMI or different concentrations of compstatin, C3aR-SAG, SB290157, PMX53, anti-C5aR2 antibody or $A8^{\Delta 71-73}$. The whole blood samples were then infected with a final concentration of 10^6 CFU/ml of *Nme*. All samples were incubated for 30 min rotating at 37 °C, serial diluted and plated for CFU measurements. Different amounts of CFUs among the different blood donors, displays differently responding individuals for each compound. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

All in all, the human whole blood experiments showed that anaphylatoxins, their receptors and also cellular processes are closely entangled. The used compounds in these experiments had no effect on the viability of *Nme* and therefore, the experiments display the direct results of blocking the complement or the ATRs. Blockade of C3aR, C5aR1 or C5aR2 revealed that important defense mechanisms are impaired without their activation. This was seen for the oxidative burst or the secretion of the cytokine IL-8. In contrast to that, the degranulation seemed to be only dependent on C5aR1 or simultaneous blockade of both C5aRs. Here the experiments might show a difference between the two receptors C5aR1 and C5aR2. Phagocytosis in contrast to that, was only altered when C3aR or the whole complement system was blocked and was not dependent on C5aR1 or C5aR2 signaling.

3.6. *Ex vivo* analysis – Possible differences in ATR signaling pathways

3.6.1. ERK1/2-phosphorylation in murine macrophages lacking ATRs

The lack of C5aR1 and C5aR2 was beneficial for the outcome of meningococcal sepsis in mice. The results could be reproduced by directly targeting C5aR1 or C5aR1 and C5aR2 simultaneously with antagonists *in vivo*. However, the underlying explanations are still not clear, as the C5aR1 is a G-protein coupled receptor, in contrast to the C5aR2. Because of a mutation in the DRY motif, the C5aR2 cannot signal via G-proteins (75, 84). Yet, C5aR2 might signal through other mechanisms, such as ß-arrestin translocation (86). ERK1/2 plays a role in ß-arrestin signaling (80) and therefore ERK1/2 phosphorylation was analyzed to find a possible common mechanism of the two C5aRs. ERK1/2 is an important signaling molecule responsible for many cellular responses and might be addressed through the C5aR1 and C5aR2 receptors, even though still under debate (81, 82, 165). In the depletion experiments, to analyze possible cellular involvements in fighting off meningococcal infections, the absence of monocytes/macrophages yielded the strongest impact on the survival of the meningococcal infected mice. Therefore, experiments were designed, in which bone marrow cells were harvested from WT, *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice. These bone marrow cells were further cultivated with L929-conditioned medium into macrophages. Those different macrophages were then infected/treated with *Nme*, C5a, *Nme* together with C5a or PMA. Upon this stimulation, ERK1/2 phosphorylation was measured via Western blot. The results of these analysis are depicted in Fig. 14. The positive control, measuring the total amount of ERK1/2, showed the presence of ERK1/2 in all samples (Fig. 14A right panel). Measuring the amount of phosphorylated ERK1/2 yielded quite diverse and interesting results (Fig. 14A left panel). Stimulation of macrophages with C5a alone yielded ERK1/2 phosphorylation within 5 min for WT and C5aR2 knock-out macrophages, but a significantly reduced signal for C5aR1 knock-outs macrophages compared to WT macrophages. Fifteen- and 30 min post stimulation, the phosphorylation was on a similarly low level for all three different macrophage populations. In contrast to that, stimulation with *Nme* did not show a phosphorylation before 15 min in the different groups. The signal was on a similarly level among the groups for all time points, with a peak at 15 min. Stimulation of the macrophage population with C5a and *Nme* simultaneously, yielded surprising differences already within 5 min. The WT signal was significantly increased with this combinational stimulation compared to C5a or *Nme* single stimulation. Vice versa, the phosphorylation in *C5ar1*$^{-/-}$ or *C5ar2*$^{-/-}$ macrophages was significantly reduced compared to the WT macrophages. Fifteen- and 30 min after stimulation the signal was on a similarly high level

for all three conditions. This effect could even be reproduced with the usage of $A8^{\Delta 71-73}$, blocking C5aR1 and C5aR2 simultaneously. The positive control with PMA showed a strong signal already within 5 min, which was slightly reduced 15- and 30 min post stimulation. All together, these experiments showed that C5a stimulation alone yielded an early response, which quickly diminished again without further stimuli. In contrast to that, the stimulation with *Nme* alone generated a later response but a more persistent one. Co-stimulation with C5a and *Nme* revealed that the early signal by C5a is mediated through C5aR1 and C5aR2. This highlights a common reaction of C5aR1 and C5aR2 in early macrophage signaling and could explain the ameliorated outcome of meningococcal sepsis in *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice. Which could mean that without the co-stimulation of either C5aR receptor, an immune reaction is elicited, but slightly delayed and maybe more controlled. This could be more beneficial than a quickly induced overwhelming immune reaction.

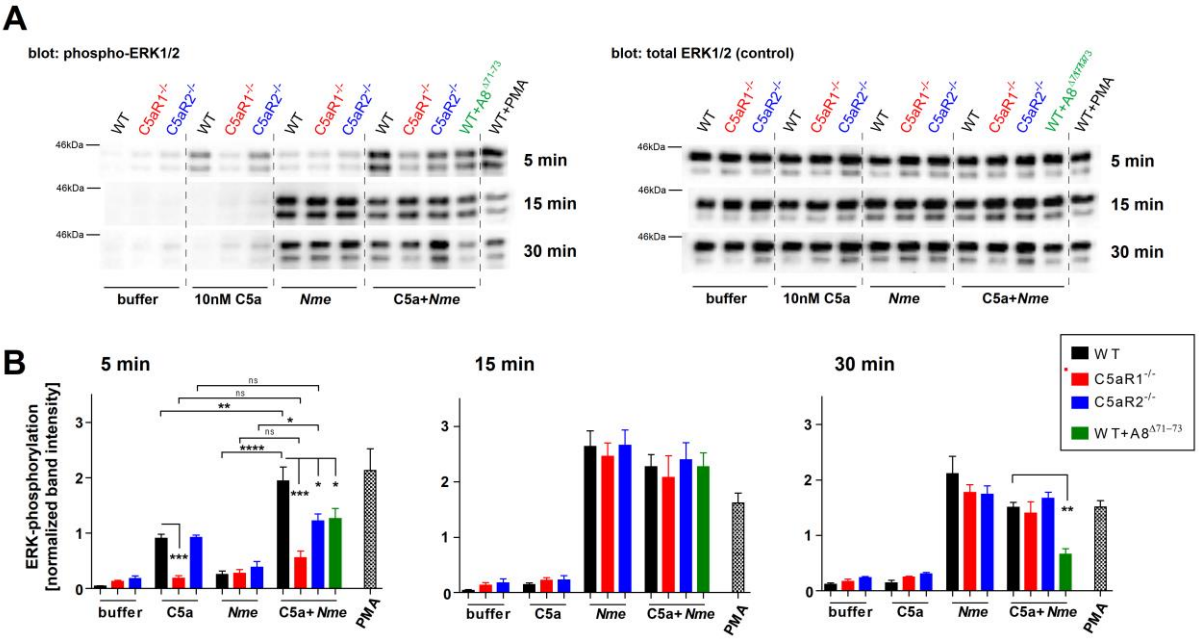

Figure 14: ERK1/2 phosphorylation in macrophages, cultivated from murine bone marrow cells, upon stimulation with *Nme*, C5a or *Nme* together with C5a

Bone marrow cells were isolated from WT, *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice and cultivated with L929-conditioned medium for 8 days into macrophages. Their phenotype ($CD68^+CD11b^+/F4/80^+$) was controlled via FACS staining. The macrophages were stimulated with murine C5a at a final concentration of 10 nM, 10^8 CFU/ml heat inactivated *Nme*, both or with PMA as a control for 5, 15 or 30 minutes. In addition, WT macrophages were treated with $A8^{\Delta 71-73}$ to block C5aR1 and C5aR2. Afterwards, the samples were harvested, the phosphorylation of ERK1/2 (**A** left panel) and the total amount of ERK1/2 (**A** right panel) was measured via Western-Blot. Summarized densitometric analysis of four independent experiments are shown below (**B**). ns, not significant; *, P<0,05; **, P<0,01; ***, P<0,001; in one-way ANOVA with Bonferroni's *post hoc* test. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

3.7. *Nme* in mucosal colonization

3.7.1. Minor impact of C3aR, C5aR1 and C5aR2 on mucosal colonization by *Nme* in CEACAM-1-humanized mice

Nme express several adhesion molecules and its believed that Opa, Opc and Pili are the most important once (100). Of those three adhesion molecules, Opa binds to the vastly on human epithelial and endothelial cells expressed CEAMCAMs (100). Johswich *et al.* studied the binding of Opa to the different human CEACAMs in a humanized mouse model. They could show that human CEACAM-1 expression was sufficient for attachment of *Nme* to human epithelial cells and that due to this attachment, the mice were able to develop an immunity towards meningococci (101). With this successfully established mouse model, they further analyzed a possible role of the complement system on the meningococcal mucosal colonization by administering CVF (101). CVF binds the complement factor B and forms a C3/C5-convertase, which is able to cleave C3 and also C5 of the complement system. As the enzyme CVF,Bb is very resistant to normal complement regulatory molecules, it continuously cleaves C3 and C5. Thereby, high levels of anaphylatoxins are released and ultimately the complement system is completely depleted (166). Using CVF in their huCEACAM-1 model right before infection, Johswich *et al.* could show a decreased colonization of their mice, indicating an involvement of the complement in mucosal colonization, upon a strong activation (101). Due to these published observations, intranasal infection experiments with CEACAM1-humanized mice were conducted, to further analyze the importance of the complement system and whether anaphylatoxins could play a role in mucosal colonization. Mice expressing huCEACAM1 and simultaneously lacking either C3, C5, C3aR1, C5aR1 or C5aR2 were challenged intranasally with Mc58. Cohorts of mice were euthanized 1 day, 3 days or 14 days post infection. Samples were washed and swabbed from their nasal cavities and analyzed for the amount of bacteria being recovered. Fig. 15A displays the number of bacteria being recovered from the different time points. 1-day post infection, all sacrificed mice showed a successful colonization by Mc58. The *huCEACAM1-C5ar1*$^{-/-}$ mice seemed to have a lower colonization rate compared to the other groups, but this difference was not significant. Three days post infection the colonization rate was again very homogenous among the different groups and in each group some mice were already not colonized anymore. Fourteen days post infection, the colonization rate dropped even further and more than half of the mice from each group were not colonized any longer. At the later time points, 3 days and 14 days post infection, it seemed as if the amount of recovered bacteria was the highest in the *huCEACAM1* mice and a bit lower in the other *huCEACAM1*

mice lacking C3, C5 or one of the three ATRs. However, this trend was not statistically significant.

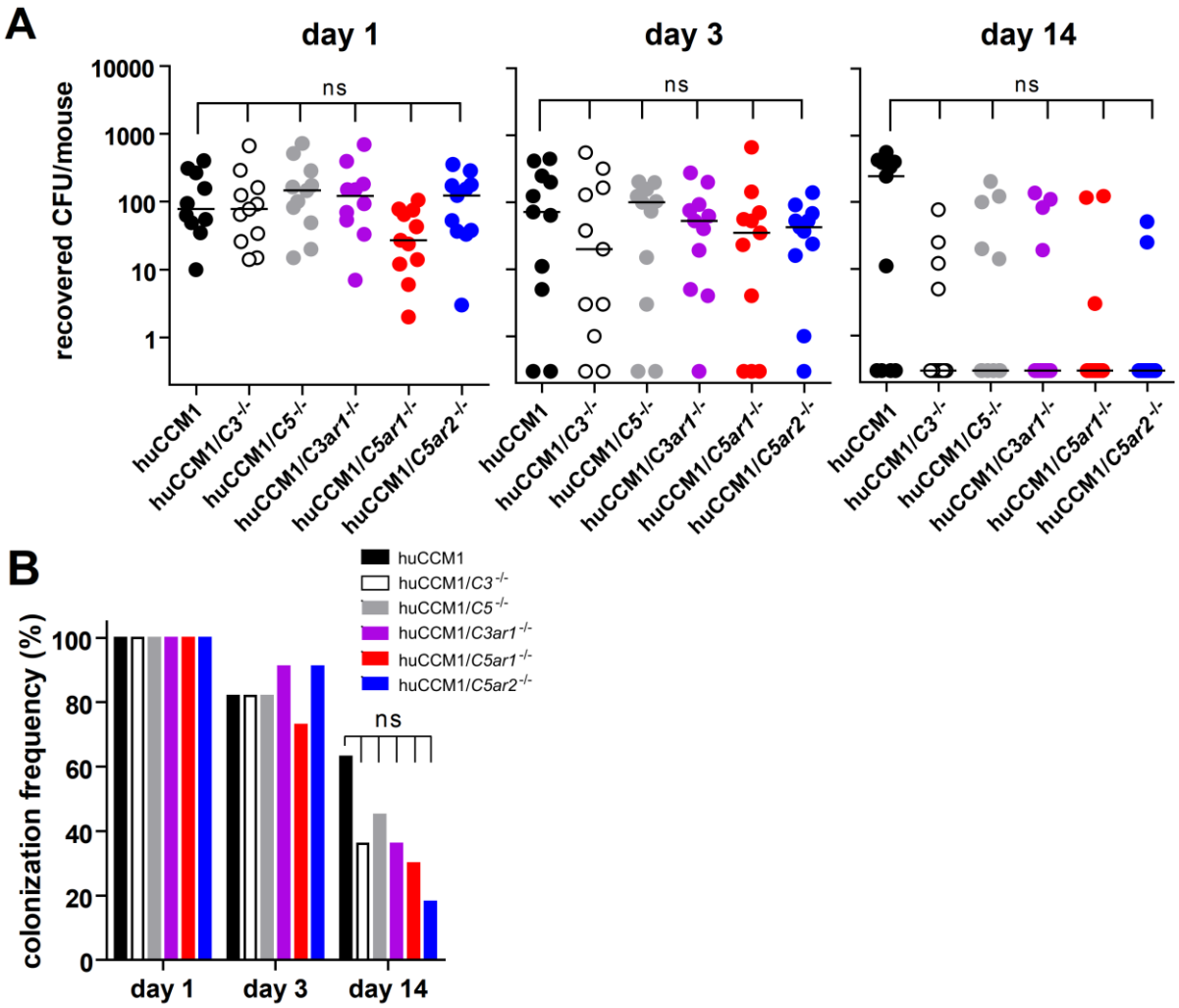

Figure 15: Mucosal colonization in *huCEACAM1*-mice lacking complement factors or ATRs
huCEACAM1-C3$^{-/-}$, *huCEACAM1-C5*$^{-/-}$, *huCEACAM1-C3ar*$^{-/-}$, *huCEACAM1-C5ar1*$^{-/-}$, *huCEACAM1-C5ar2*$^{-/-}$ mice were infected i.n. with 10^5 CFU of *Nme*. Mice were sacrificed 1-, 3- or 14-days post infection. Postmortem, nasal swab and wash samples were taken for accessing the bacterial load at the indicated time points as displayed in figure **A**. Figure **B** shows in percentage how many mice in each group were still colonized at the different time points post infection. ns, not significant; in one-way ANOVA with Bonferroni's *post hoc* test. The data were published in Muenstermann *et al.*; 2019; Virulence (157).

Fig. 15B shows the colonization frequency of mice for each genotype. The colonization rate was still as high as 80% 3- days post infection in all mice and then dropped quickly below 50% for all genotypes, except for the *huCEACAM1* mice, which was still above 60%, but again not significantly different from the other groups. All in all, it can be said that the complement factors C3 and C5 do not directly impact the colonization rate. Additionally, also the lack of

one of the ATRs did not change the outcome of the experiment significantly. Whereas the complement and especially the ATRs play an important role in IMD and clearance of meningococci, there is no evidence that the complement factors C3, C5 or either of the ATRs plays an important role in the nasal colonization.

3.8. The type II CRISPR/Cas system in mucosal colonization

3.8.1. Minor role of type II CRISPR/Cas system in meningococcal mucosal colonization in huCEACAM-1 expressing mice

Nme is a successful avirulent commensal bacterium in the nasopharynx of approximately 10% of the German population, according to the RKI (28), and in 8-25% of the world's population (26). To accomplish these high carrier rates, *Nme* express an array of different adhesins and other molecules aiding them in protection from the different human immune mechanisms present (167). Another system that could be beneficial for *Nme* in nasopharyngeal colonization is the type II CRISPR/Cas system. Sampson and colleagues found a reduced virulence of cas9 *Nme* mutants in adhesion assays with adenocarcinoma alveolar basal epithelial cells (119). Heidrich *et al*. showed an increased virulence of the *Nme* strain 8013 in dependence on the type II CRISPRC/Cas system in cell culture adhesion assays, with an nasopharyngeal epithelial cell line (121). Therefore, in cooperation with the group of Prof. Christoph U. Schoen a possible involvement of the CRIPSPR-Cas system was tested *in vivo*. A possible enhancement of *Nme* mucosal colonization was analyzed with different meningococcal mutants in the huCEACAM-1 mouse model. Mice expressing huCCM1 were infected intranasally with the *Nme* WT-strain 8013 and different CRISPR/Cas mutants. The inocula contained in a ratio of 50 to 50 the WT strain and either one of the following 8013 mutants: Δ*cas9* clone 1, Δ*cas9* clone 2, Δ*trc*, a complementary strain of those three strains or the double deletion mutant ΔΔ*rcoF1/2*. Cas9, encoded by the gene *cas9*, and the tracrRNA, encoded by gene *trc*, are important factors in the type II CRISPR/Cas system. The crRNA, coding for the foreign DNA sequence, does not need to undergo maturation in *Nme* and can directly be tagged by the tracrRNA. This cRNA:tracrRNA complex can then be bound by the endonuclease Cas9. Cas9 is the central protein of the type II CRISPR/Cas system. It scans the DNA for the specific target sequence and mediates the cleavage. Both tracrRNA and Cas9 play a pivotal role in the type II CRISPR/Cas system, which is impaired in the respective knock-out mutants Δ*cas9* and Δ*trc* (110, 121).

In addition to the type II CRISPR/Cas system, a possible involvement of the two trans-acting small RNAs (sRNAs) RcoF1 and RcoF2 (RcoF1/2) on nasopharyngeal colonization was tested. RcoF1/2 are encoded by the genes *rcoF1* and *rcoF2*. In an Hfq-dependent manner, RcoF1/2 is involved in degradation of the *prpB* mRNA. PrpB is a possible colonization factor in *Nme*, which is upregulated in the double knock-out mutant strain ΔΔ*rcoF1/2* and could give the

meningococci an advantage in nasopharyngeal colonization (131). The results of the *huCEACAM-1* mice infection with the different strains are displayed in Fig. 16.

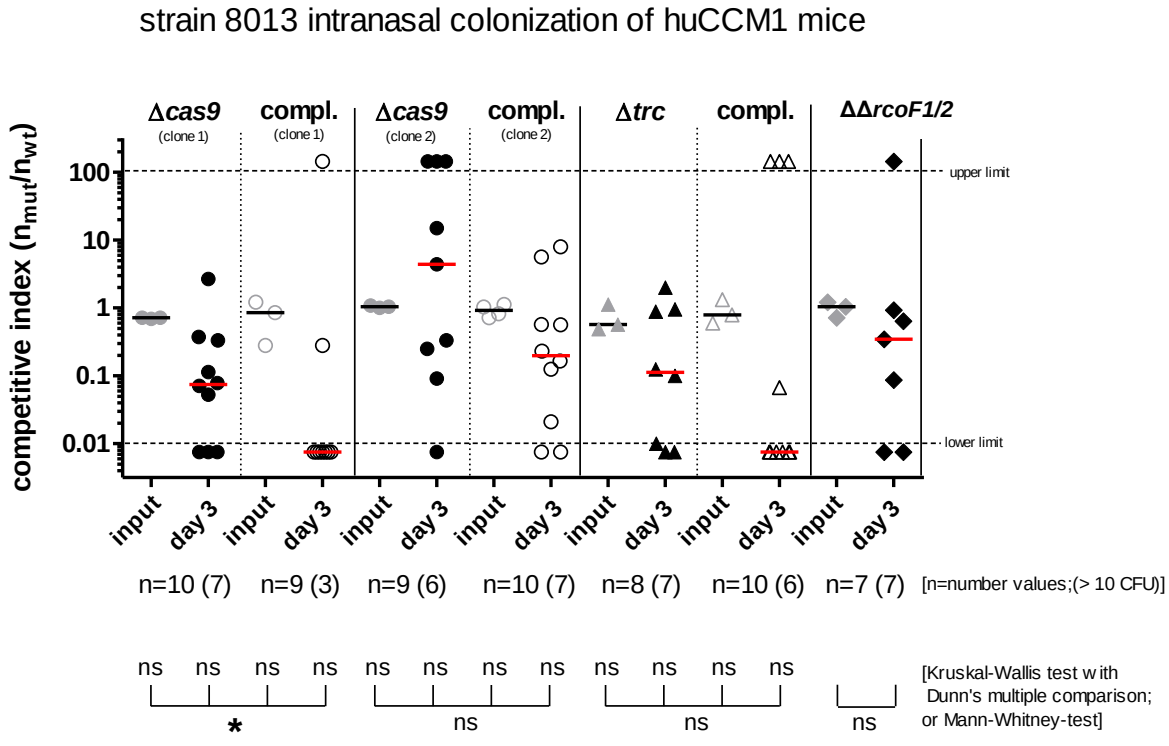

Figure 16: Mucosal colonization of CRISPR/Cas *Nme* mutants in *huCEACAM1*-mice
Mice expressing huCEACAM1 were intranasally challenged with 5 µl of a final concentration of $2x10^{10}$ CFU/ml of *Nme*. Each mouse was infected with an inocula of the *Nme* WT strain 8013 mixed together at a ratio of 50 to 50 with one of the following 8013 mutants: Δ*cas9* clone 1, Δ*cas9* clone 2, Δ*trc*, a complementary strain of those three strains or the double deletion mutant ΔΔ*rcoF1/2*. The actual ratios achieved are labeled with "input". At day 3, the mice were euthanized and recovered meningococci from their nasal cavities were plated on chocolate agar plates for an overnight culture. Up to 100 clones were then transferred onto chocolate agar plates with or without kanamycin and incubated overnight. This figure displays the ratio of the competitive index of recovered kanamycin resistant mutants to kanamycin sensitive WT bacteria. N shows the number of animals, that were infected and the numbers in brackets display the number of animals, from which more than 10 CFUs were recovered. Upper and lower limits indicate a recovery rate of 100% of either mutants or WT bacteria. This study was performed blind, as the experimenter did not know which clone had which mutation. ns, not significant *, $P<0,05$; Multiple comparisons were done using the Kruskal-Wallis test with Dunn's multiple comparison. Comparisons of two groups were done using the Mann-Whitney-test.

Two different clones of the *cas9* mutant were used for the i.n. infection and both yielded different results. Clone 1 of those mutants seemed to have a reduced fitness, whereas clone 2 showed the opposite effect, as more mutants than WT bacteria were recovered from those infected mice. Surprisingly, the complementary strain of clone 1 showed the same reduced fitness compared to the WT clone of this mutant. The complementary strain of clone 2 on the

other hand, showed a trend of a more reduced fitness than the cas9 mutant. Next, the Δ*trc* mutant was analyzed. This mutant showed a trend to a reduced fitness compared to the WT strain. Yet, the complementary strain of the Δ*trc* mutant, which should have the same fitness as the WT strain, was quite inconclusive. The recovered bacteria were in some samples either all from the WT- or all from the mutant strain. This discrepancy with the complementary strains was surprising and could have been some sort of an experimenter bias, which however can be ruled out, as this study was performed blind and therefore it was not known before the end of the experiments, which strain was the mutated and which was the complemented one. The last strain, the double mutant ΔΔ*rcoF1/2* showed no significant changes between the WT and the mutated bacteria in mucosal colonization.

From the data, generated in the huCEACAM-1 expressing nasal infection model, the role of the type II CRISPR/Cas system could not be determined. A benefit in colonization was also not detected in the ΔΔ*rcoF1/2* mutant. Due to the differential outcome of the WT bacteria and the complementary strains, further experiments with this model would not seem to be very prosperous.

4. Discussion

Nme, is the causative agent of invasive meningococcal sepsis. This life-threatening pathological condition causes around 200 deaths per year in Germany, with a very high mortality rate of over 10% even with intensive care treatment (28). These bacteria are commonly found as commensals in the upper respiratory tract of their sole host, humans and is carried by approximately 8-25% of the world's population (26). They are well adapted to their host, express a shielding capsule (167) and are even able to protect themselves from complement mediated killing by recruitment of inhibitory proteins, masking themselves as host cells (57). Many different mechanisms of these bacteria-host interactions are still unclear and their better understanding could help to increase the survival rates of patients suffering from meningococcal sepsis. In the framework of this thesis, experiments were conducted to shed further light on the different cellular roles in IMD and the roles of the three anaphylatoxin receptors C3aR, C5aR1 and C5aR2 in IMD, in cellular immune responses and in mucosal colonization. In addition, a possible growth advantage in mucosal colonization by *Nme* depending on the type II CRISPR/Cas9 system was analyzed.

4.1. A cellular involvement in meningococcal sepsis was confirmed by cellular depletion

It has long been established, that the complement system plays the most important role in fighting off IMD, as infections with *Nme* are strongly increased in patients with complement defects. This is most pronounced in patients with a defect in the late complement component C7, where the risk to get IMD is increased by striking 10.000-fold (47). Because the complement system is the most important defense mechanism against *Nme*, it has long been questioned whether, and to what extent, other immune reactions mediated by cellular components might play a part in fulminant sepsis. In infections with other encapsulated bacteria like *S. pneumoniae*, which can also cause meningitis and fulminant sepsis, it has already been shown that in addition to the complement system (168) also cellular responses are important in fighting off an infection (152). Additionally, in meningococcal sepsis in mice, the outcome greatly depends on the expression of C5aR1, as shown by Herrmann *et al.* (93). The anaphylatoxin receptors C3aR, C5aR1 and C5aR2 are expressed on several different immune cells, among them are mast cells, neutrophils and monocytes/macrophages (74). This fact and their important functions in IMD, indicates to further cellular contributions in fighting off

meningococcal infections. Therefore, murine *in vivo* experiments were designed, in which these cells were directly targeted by depletion or stabilization.

Mast cells respond extremely fast to invading pathogens and can be directly activated via antigen detection by IgE, IgG or TLRs (61) or via anaphylatoxins stimulation upon complement activation (153). Mice in these experiments had not been challenged previously with *Nme*, therefore an activation via IgE or IgG would be most unlikely. Naive immune cells can recognize pathogens by the expression of PRRs, like TLRs for example, which detect PAMPs (40). Mast cells express among others TLR4 and can be stimulated via LPS from *Escherichia coli* to produce different cytokines (169). TLR4 in macrophages is known to detect also LOS from *Nme* (170), which lets one speculate, that also mast cells might be activated via this pathway in IMD. Furthermore, mast cells can be activated by the release of C5a, highly present in IMD (93). As mast cell could be activated by *Nme*, they might also play a role in meningococcal infections. However, stabilization experiments done in this work and Johannes Herrmann's thesis (156), published together in Herrmann *et al.* (93) yielded, that mast cell do not play an important role in meningococcal sepsis in this murine model.

Stabilization of mast cell was done by the administration of cromolyn, which was first established as an asthma therapeutic, because of its ability to stabilize mast cells (171). After discovery, cromolyn has been used to study different functions of human- (172) and rat mast cells (173). In addition, it was also used to investigate systemic effects of cromolyn administration in mice (174). Whereas effective stabilization of mast cells has been reported in human and rat mast cells, there has been some doubt whether cromolyn acts in the same way on mice mast cells. Findings suggest, as higher concentrations are needed in mice, that cromolyn might not be stabilizing mice mast cells in the way it does in humans. In fact, cromolyn might even act on other cells, as Oka *et al*. showed a reduction of TNF secretion in mast cell knock-out mice upon cromolyn administration (175). This could mean that a possible protective role of mast cells in meningococcal sepsis might not have been detected with this murine mast cell stabilization model. To analyze the exact role of mast cells in IMD, C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice, lacking mast cells, could be used instead of cromolyn (175).

Analyzing the murine findings in regard to a human setting is difficult, as mast cells could play a role in humans, even though not seen in these experiments. Mast cells are strongly activated via bound IgE, which is an important mechanism in allergic reactions, but not relevant for bacterial infections (40, 61, 172). A weaker activation of mast cells can also occur via bound IgG (63). Mice in these experiments were not infected with *Nme* previously and so a possible

activation via IgG would not have been taken place in this murine model, but could play a role in humans, as asymptomatic carriers develop antibodies against meningococci (176). All in all, this murine infection model with cromolyn mast cell stabilization did not show a protective or adverse effect of mast cells, but cromolyn might not have worked in the anticipated way. Therefore, it cannot be entirely ruled out that mast cells do play a role in IMD.

In contrast to mast cells, the lack of neutrophils or monocytes/macrophages significantly decreased the survival of mice compared to the control group. Most mice in which neutrophils or monocytes/macrophages were depleted reached the humane endpoint already within 24 h post infection, even with this nonlethal dose of *Nme*. Surprisingly, the 12 h blood samples for bacterial burden assessment revealed a strong significant increase in the *Nme* blood concentration in monocyte/macrophage depleted samples compared to the control group, but not in neutrophil depleted mice, even though both groups showed a similarly low survival rate. This could have different explanations. Neutrophils are most efficient in clearing bacterial infections by phagocytosis and oxidative burst and surpass monocytes/macrophages in this sense, as they are recruited in great numbers to sites of inflammation within hours (40), as shown in wound healing models (177). This recruitment is even further increased with pathogenic stimuli present (178). In the wound healing model from Agaiby and Dyson, the increase in monocyte/macrophage numbers peaked at 24 h post stimulation, but was at a stable level for several days, compared to neutrophil numbers, which dropped quickly in that model (177). A strong neutrophil mobilization is seen early on in human *Nme* patients, which results in a high white blood cell count or also in neutropenia, if the neutrophils are rapidly recruited into infection sites (179). In meningococcal infections with meningitis, a strong influx of neutrophils into the cerebrospinal fluid is characteristic (180). This lets one wonder, why the bacterial numbers were then higher 12 h post infection in monocyte/macrophage depleted mice and not in mice where neutrophils were depleted. An explanation could be that monocytes and especially resident macrophages, which are already at the infection site, are important for the early reactions against *Nme*. The bacterial concentration in mice with an intact monocyte/macrophage population, but depleted neutrophil population, was on a similar level as seen in WT mice 12 h post infection, showing that monocytes/macrophages are capable of keeping the numbers of *Nme* at bay at early time points. After 12 h post infection, their abilities to keep the *Nme* level low or at a constant number seems to have reached its limit. This highlights the importance of infiltrating neutrophils upon pathogen detection, as most mice reached the humane endpoint before 24 h without neutrophils present. Vice versa, as the neutrophils are important in fighting off IMD at time points between 12 h and 24 h, they are

not capable of eradicating *Nme* without the initial monocyte/macrophage responses, displayed in a significant increased bacterial burden already 12 h post infection in mice without monocytes/macrophages. This whole cascade of cellular responses seems to be completely thrown off without monocytes/macrophages as these mice, even though neutrophils are present, reach the humane endpoint already very earlier with this otherwise nonlethal dose of *Nme*. These results show, on one hand, that also in IMD cellular components of the immune system are very important in fighting off *Nme*, as it has also been seen in *S. pneumoniae* infection (152) and that the complement system is not capable of clearing the infection in this murine experimental induced meningococcal sepsis model alone. On the other hand, these data also show a great interplay of monocytes/macrophages and neutrophils in IMD, as neither of the cells is capable of clearing the infection without the other one. Nevertheless, what this interplay consists of and where the tipping point for this system lays, needs to be further analyzed. The reason for neutrophils not being capable to fight off IMD alone could either be that not enough neutrophils are recruited, that they are recruited to late or that they are not recruited at all to combat meningococci. Another point to consider is that Neutrophils were depleted with the RB6-8C5 antibody. RB6-8C5 is an antibody that was originally thought to only target neutrophils and was used for depletion experiments (181). The antibody targets Ly6G, which is specifically expressed on neutrophils, but also Ly6C, which is expressed on neutrophils, but also on some monocytes and macrophages (182, 183). Therefore, it cannot be ruled out that the effects seen after neutrophil depletion, to some extent might also come from a partial depletion of certain subtypes of monocytes or macrophages.

Many cellular processes are entangled with the ATRs and especially C5aR1 showed detrimental effects in murine IMD (93). This led to speculations, whether this beneficial effect might also recover the reduced survival rates in monocyte/macrophage or neutrophil depleted mice. Surprisingly, the results from the depletion experiments were almost the same in C5aR1 knock-out and WT mice. Most *C5ar1*$^{-/-}$ mice succumbed to this otherwise nonlethal dose, when either of the population was depleted. However, the overall survival was slightly increased compared to untreated mice. Also, the bacterial burden was significantly increased 12 h post infection, when monocytes and macrophages were depleted, compared to untreated mice and 18/24 h post infection significantly increased in monocytes/macrophage and neutrophil depleted mice. These data show that a successful immune reaction must be on a cellular level so severely disrupted, that a possible beneficial absence of C5a mediated signaling is not overweighing the detrimental effects from the depletion of either monocytes/macrophages or neutrophils. Interestingly, there was a slight increase in the survival rates and also the bacterial burden in

the different mice showed a trend to a slight increase compared to WT mice. Whether this is however some experimental artefact, if C5aR1 knock-out mice are able to bear higher concentrations of *Nme* or if *Nme* are faster growing in the absence of C5aR1 signaling would have to be further researched on. The direct interplay between monocytes/macrophages and neutrophils is also important in other disease models like systemic *S. pneumoniae* infection (152). Therefore, it would be interesting to further analyze, what happens in IMD without monocytes/macrophages. Are the neutrophils not recruited or not stimulated? Analyzing the amount of recruited neutrophils to the peritoneum could be measured with a peritoneal lavage and measured via flow cytometry analyzes. This would be, however, not very easy to measure, as the biosafety concerns of this procedure would be high. Additionally, it is to be expected that the recruited neutrophils would undergo NETosis (184), which would result in cellular clumping and could make further analyzes like flow cytometry, which is sensitive to cell sizes, very difficult. This could change the results, as the neutrophils would vary in size and many cellular aggregates would affect the analysis. Therefore, further cellular process analysis, such as the cytokine profile in IMD infection would be the most suitable cause of action, to further analyze this interplay.

The depletion experiments with *C5ar1*$^{-/-}$ mice did not change the outcome of the sepsis compared to WT mice. Most likely this means, that the positive effects of the lack of C5aR1 expression, as seen in meningococcal sepsis with a lethal dose of *Nme*, is outweighed by the complete loss of either neutrophil or monocyte/macrophage population. The C5aR1 is an important signaling molecule in neutrophils and in monocytes/macrophages for the oxidative burst and the degranulation, as shown *ex vivo* (74). Therefore, it would be very interesting to analyze the role of C5aR1 specifically in neutrophils or macrophages *in vivo*, without the depletion of the whole population. This could be accomplished with floxed GFP-C5aR1 knock-in mice. By mating them with LysMcre mice, the receptor would be specifically blocked in neutrophils and macrophages (185). This would then allow the direct study of these still functional, but devoid of C5aR1 signaling, cell populations *in vivo*.

All in all, these data underscore that in addition to the complement system also cellular involvement plays an important role in the pathophysiology of IMD. Mice with intact complement systems, but without monocytes/macrophages or neutrophils showed a significantly decreased survival rate, when challenged with a for untreated mice nonlethal dose of *Nme*. Undoubtably, the complement system is important in IMD, as mice deficient for C3 already reached the humane endpoint with injections of as little as 10 CFU of *Nme* (93), but on

the other side, WT mice without cellular components of the innate immune system are also not capable in surviving IMD. The underlying cellular mechanisms, especially in regard to the anaphylatoxins, were further evaluated in this thesis.

4.2. The involvement of C3aR, C5aR1 and C5aR2 in murine IMD

ATRs are entangled in many processes from the immune system and can even have detrimental effects as shown for C5aR1 in IMD (93). The data of depletion experiment from this thesis and Johannes Hermann's thesis (156), published in Hermann *et al.* (93), showed that not only the complement system is of utmost importance in fighting off IMD, but also that the interplay of monocytes/macrophages and neutrophils is essential, in which the anaphylatoxin receptor C5aR1 must play a detrimental role (93).

Seeing this important role of C5aR1, the question how C3aR and C5aR2 might be involved in IMD came up and was analyzed by injection of mice with a lethal dose of *Nme*. In line with the published results from Herrmann *et al.* (93) also in this thesis, C5aR1 mice showed a significant increase in survival compared to WT mice. The role of the C5aR1 in sepsis is therefore very clear, but the role of the other C5aR, the C5aR2, is still not well-defined. There is still much discussion going on about whether the C5aR2 is only a non-signaling scavenger receptor (85) or could be involved in inflammatory responses, as shown by Rittirsch *et al.* in a CLP model (92). This thesis confirms that C5aR2 plays an important role in IMD beyond being just a scavenger receptor. The survival rate of mice lacking C5aR2 was significantly increased, compared to WT mice, upon challenge with a lethal dose of *Nme*. Underlined were these findings with a significantly decreased clinical score in *C5ar1*$^{-/-}$ mice from 12 h on and in *C5ar2*$^{-/-}$ mice from 18 h on post infection. In contrast to the two C5aRs, C5aR1 and C5aR2, the lack of C3aR expression had a detrimental effect on the outcome of the infection. With this lethal dose of *Nme*, *C3ar*$^{-/}$ mice reached the humane endpoint even earlier than WT. This ameliorated disease progression in C3aR knock-out mice was also confirmed with a significant increase in the clinical score 18 h post infection. The bacterial burden was significantly reduced in C5aR1 and C5aR2 knock-out mice 3 h, for *C5ar1*$^{-/-}$ 12 h and for *C5ar2*$^{-/-}$ 18-27 h post infection. On the other hand, a change in the bacterial burden was not visible between WT and *C3ar*$^{-/-}$ mice. These results show that in IMD not only the C5aR1, but also the C5aR2 play an important role. In contrast to that, the C3aR seems to be of rather protective nature in IMD. This was, however, not visible in changes in the bacterial burden, which shows that underlying mechanisms beyond the mere killing of bacteria must be altered in the absence of C3aR. Seeing

this detrimental effect of C3aR absence raised the question, whether a nonlethal dose of *Nme* in mice would show similar effects. With a nonlethal dose of *Nme*, some C3aR knock-out mice still reached the humane endpoint, but the survival was not significantly decreased. There was some increase in the clinical scoring 12 and 24 h post infection and an increase in the bacterial burden in mice lacking C3aR 12 h post infection. However, as the survival rate was not significantly reduced, these findings show that for a lethal dose of *Nme*, C3aR expression is important, but the underling mechanisms impaired without C3aR do not seem to be necessary in eradicating nonlethal infections.

Anaphylatoxin receptors are expressed on several different cell types and play an important role in many different processes (74). To further analyze these processes samples for cytokine analyses were taken, to see whether the absence of ATRs might be visible in different cytokine profiles. Analysis of ELISA samples taken 12 h post infection, when the infection was at its peak, revealed that the cytokine levels for all tested cytokines showed a trend to a lower level in all three ATR knock-out strains compared to WT mice. Significant reduction was seen for CXCL-1 and IL-6 for all three ATRs and, in addition to that, the cytokine levels for TNF-α, IFN-γ and MCP-1 were significantly reduced only in mice lacking C5aR1. TNF- α and IL-6 are pro-inflammatory cytokine released early in infections, by macrophages and monocytes (68, 69). A reduction of IL-6 levels in ATR knock-out mice could mean that the initial inflammation is less severe. TNF-α in addition is also secreted early on and is decreased significantly in C5aR1 knock-out mice, which could explain the slightly higher survival in *C5ar1*$^{-/-}$ mice compared to *C5ar2*$^{-/-}$ mice. In line with that, attraction of neutrophils to clear the bacterial infections seems to be lessened in all three anaphylatoxin knock-out mice strains, as CXCL-1 in all and MCP-1 in C5aR1 knock-out mice are significantly reduced. These observations seem to be positively affecting the outcome of IMD, which underlines the notion, that the immune system is not only beneficial, but could also cause some of the complications seen in sepsis patients (51). In fact, Younger *et al.* observed in rats, when the complement was excessively activated via CVF injection, symptoms of shock that can also be seen in sepsis. This highlights the double edged sword capacity of the complement system and especially the anaphylatoxins (91). Surprisingly, these reduced responses in cytokine secretion were also seen in C3aR knock-out mice. This could have different explanations. For example could it mean that all ATRs are to some extent involved in the secretion of pro-inflammatory cytokines, by direct or indirect means, but the mechanisms that are finally causing the ameliorated or worsened outcome in mice, are not dependent on the cytokine secretion. Another explanation could be that the release of C5a at some stage might not be beneficial anymore. Huber-Lang *et al.* reported that too high

concentrations of C5a reduce phagocytosis and oxidative burst in neutrophils, which could negatively affect the clearing of bacteria in sepsis (186). This effect would be in line with the observation that cytokine levels are similar in all three ATR knock-out mice. It could mean that at later stages something goes wrong, as mice survive when the C5a signaling is impaired, compared to C3aR knock-out mice, where this detrimental signaling via C5aR1 and C5aR2 is still active. In line with that, cytokine analysis 3 h post infection revealed that CXCL-1 secretion was already significantly reduced in ATRs knock-out mice compared to WT mice. Further suggesting, that a milder course of immune reaction is important, but reduced recruitment of neutrophils, does not suffice, but rather is needed in combination with a reduced activation of immune cells via C5a through C5aR1 and C5aR2.

4.3. ATRs as a possible therapeutic target *in vivo*

C5aR1 knock-out mice showed a significantly increased survival in IMD and Herrmann *et al.* could even reproduce this effect by targeting C5aR1 in WT mice with the compound PMX205, which inhibits C5aR1 (93). Seeing the beneficial effect in C5aR2 lacking mice and the detrimental effect of C3aR lack in mice raised the question, whether these two receptors could also be used as therapeutic targets. WT mice were injected with lethal doses of *Nme* and treated with C5aR1, C5aR1/C5aR2 or C3aR1 blocking agents or with a C3aR activating agent. Blocking of C5aR1 with PMX205 showed the same beneficial effect as seen by Herrmann *et al.* (93). Targeting C5aR1 and C5aR2 simultaneously with $A8^{\Delta 71\text{-}73}$ yielded similar significantly increased survival rates as seen in mice, where C5aR1 was blocked with PMX205. There is currently no reagent available which blocks C5aR2 in mice (84) Therefore, this increased survival cannot be associated only to C5aR2, as it could also be mainly come from blocking C5aR1. Nevertheless, these results show that blocking C5aR2 is not negatively affecting the outcome, which would be in line with the *C5ar2*$^{-/-}$ mice experiments.

Blocking C3aR was not significantly increasing the survival rate, however more mice survived in this group, than in the IMD experiments with *C3ar*$^{-/-}$ mice. There is actually the possibility that SB290157 is not only working as an antagonist (158), but also as an agonist and therefore a complete blockade of the C3aR might not have been reached. This would explain why the deleterious outcome in *C3ar*$^{-/-}$ mice was not seen, when the C3aR was targeted with SB290157. Vice versa, as the absence of C3aR in the knock-out mouse strain was detrimental in IMD, a positive effect might have been accomplished with activation of this receptor by the

superagonist (WWGKKYRASKLGLAR) (144). Upon using this superagonist *in vivo*, no changes in the survival rate were detected. In line with the survival curves, the highest changes were seen for the bacterial burden and cytokine secretion, when one of the C5aRs was targeted. The bacterial burden was significantly decreased in mice 12 h and 18-27 h post infection, when C5aR1 alone or both C5aRs were targeted. Blocking both C5aRs with $A8^{\Delta 71\text{-}73}$ even resulted in a significantly reduced bacterial burden as early as 3 h post infection. Furthermore, a significant reduction was seen for the pro-inflammatory cytokine IL-6 and the neutrophil chemoattractant CXCL-1 12 h post infection, when C5aR1 alone or both C5aRs were blocked. Interestingly, the blockade of both C5aRs with $A8^{\Delta 71\text{-}73}$, when compared to the blockade of C5aR1 alone with PMX205, yielded a significantly lower bacterial burden already 3 h post infection and significantly lower IL-6 and CXCL-1 levels 12 h post infection. Seeing a better clearance of bacteria, while also lower cytokine levels are detected, might suggest that a less severe immune reaction is beneficial in fighting off *Nme*. In the future, when a specific C5aR2 blocking reagent is available for mice, it would be interesting to test, whether a double blockade of C5aR1 and C5aR2 is better than a single blockade of each C5aR for treatment. These *in vivo* data show that not only C5aR1, but also C5aR2 could be a possible target for therapeutic interventions in IMD. Nevertheless, these blockades were done in an *in vivo* mouse model and would have to be tested next in a human whole blood setting.

4.4. Murine PMN responses to *Nme* are not dependent on C3aR or C5aR2, but on C5aR1 expression

The differential outcome of IMD in mice lacking either C3aR, C5aR1 or C5aR2 could have different reasons. One cell population that was important in clearing meningococcal sepsis in the murine depletion experiments were neutrophils. Mice without neutrophils showed a reduced survival rate compared to WT mice. To test for the contribution of PMNs to the pathophysiologies in the different ATRs knock-out mice, murine PMNs were analyzed for their oxidative burst, degranulation, phagocytosis, bacterial killing and IL-6 secretion in the absence of ATRs.

Even though *C3ar*$^{-/-}$ mice succumbed faster to IMD, there was no change in any analyzed response in PMNs from *C3ar*$^{-/-}$ mice compared to PMNs from WT mice. This could be explained by recent studies showing that murine PMNs might not express the C3aR (187). Therefore, these findings cannot be directly translated to a human setting.

Significant changes in the oxidative burst and degranulation could only be observed in PMNs isolated from *C5ar1*$^{-/-}$ mice. These results are in line with the data published by Herrmann *et al.* (93). The observed reduced oxidative burst and degranulation in *C5ar1*$^{-/-}$ PMNs, could mean that unwanted damage to the host would be reduced *in vivo* (188). Studies using the CLP-induced sepsis model, displayed damage in different organs such as the kidneys, liver or the lung (189). Dahlte *et al.* also observed increased levels of neutrophils in the lung and liver in their murine CLP-induced sepsis model (189). Vice versa, the important phagocytosis for bacterial clearing (40) was not impaired in any of the three different ATRs lacking PMN populations. This could mean that in meningococcal sepsis, phagocytosis by PMNs is more favorable for fighting off *Nme*, than degranulation or the oxidative burst, which could result in unwanted damage (189).

Unlike in *C5ar1*$^{-/-}$ PMNs, in *C5ar2*$^{-/-}$ PMNs there was only a trend for a reduced degranulation visible upon *Nme* stimulation, but this was not significant. None of the other responses was impaired without C5aR2 expression, which shows a functional difference between these two C5a receptors in PMNs.

The release of the cytokine IL-6 was not impaired in any of the three PMN populations and was on a similar level for all three ATRs. This means, that the reduced amounts of IL-6 observed in the *in vivo* experiments were caused by another cell type, most likely resident macrophages. In addition, experiments with PMNs from *C3*$^{-/-}$ mice showed that phagocytosis is strongly dependent on complement opsonization by C3b, highlighting the importance in such whole blood assays for an intact complement system. From all three ATRs, significantly reduced responses were only seen in *C5ar1*$^{-/-}$ PMNs, but this could be different *in vivo*. In these experiments, PMNs were activated via *Nme* and C5a released by the intact complement system. *In vivo*, the additional PMN stimulation by monocytes/macrophages or the interaction with endothelial cells could result in a different activation of PMNs, especially in the absence of C5aR signaling (71, 188).

All together, these PMN analysis confirmed previous findings from Herrmann *et al.* in *C5ar1*$^{-/-}$ PMNs (93) and showed that in contrast to that, PMN responses are not altered without C3aR or C5aR2 expression. However, the results regarding the C3aR might be different in human PMNs, as murine PMNs most likely do not express a C3aR (187).

4.5. ERK1/2 signaling impaired in *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice

The loss of either C5aR1 or C5aR2 showed a significantly ameliorated disease outcome in IMD. However, C5aR2 is unlike C5aR1 not coupled to G-proteins, because of a mutation in the DRY motif (84). Therefore, a long debate has been going on about whether C5aR2 is able to transmit signals at all or is only functional in an interplay with C5aR1 as a scavenger receptor (84). Nowadays, it is established that there is also G-protein independent signaling via β-arrestin and that GPCRs can signal thereby without being coupled to G-proteins (79). Kalant *et al.* showed in HEK cells, expressing the hC5aR2, that this could also be a mechanism by which C5aR2 signaling, independent of G-protein coupling, could happen (86). As ATRs are expressed on many immune cells and depletion experiments revealed that neutrophils were especially important for keeping *Nme* at bay *in vivo*, next, experiments were performed to analyze their role in neutrophils. These experiments showed that there are functional differences in neutrophils when C5aR1 or C5aR2 are missing, but only for the oxidative burst. The second population, which is important as seen in depletion experiment were monocytes/macrophages. Especially macrophages that are already present in tissues and more precisely their early reactions to invading pathogens must be important. Many different immune responses are mediated through different cellular pathways and one important mechanism is the phosphorylation of ERK1/2, which is also linked to C5a signaling (84). Therefore, the phosphorylation of ERK1/2 was analyzed in macrophages in relation to C5aR1 or C5aR2 expression, to shed further light on possible effects dependent on the two C5aRs. Bone marrow derived macrophages from WT, *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice were stimulated with *Nme* in combination with or without C5a. The phosphorylation of ERK1/2 was then measured via western blot analyses at different time points. These experiments showed interesting differences between the ERK1/2 phosphorylation at an early time point. Macrophages that were stimulated with C5a alone only showed a short ERK1/2 phosphorylation in WT and *C5ar2*$^{-/-}$ macrophages, which faded quickly. In contrast to that, the co-stimulation with C5a and *Nme* showed a strong phosphorylation in WT macrophages and a weaker response in *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ macrophages. The same effect could even be reproduced by blockade of C5aR1 and C5aR2 simultaneously with A8$^{\Delta 71\text{-}73}$. These results show that there is an early response to *Nme* depending on C5a in macrophages, which is significantly reduced in *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ macrophages. The literature regarding C5aR and ERK1/2 phosphorylation shows quite diverse results (81, 82, 165). The results from this thesis are in line with Hsu *et al.* who also saw an ERK1/2 phosphorylation in bone marrow derived macrophages from WT mice after 5 min, which was quickly reduced afterwards (190). In contrast to this work, Hsu *et al.* saw

only a small signal of pERK1/2 for macrophages lacking C5aR2 and stated, that it was not different than the signal from macrophages lacking C5aR1 (190). This difference is probably caused by different experimental setups. The data from this thesis show that C5a alone induces a small and short signal upon stimulation in WT mice, which is much stronger when C5a and *Nme* together stimulate macrophages. This initial strong response can be ameliorated via blocking C5aR1 and C5aR2 or is reduced in the absence of one of the C5aRs. This different initial response could be responsible for the positive outcome of IMD in *C5ar1*$^{-/-}$ and *C5ar2*$^{-/-}$ mice and would have to be investigated further. Macrophages are important in clearing *Nme* infections and this *Nme* and C5a dependent stimulation on ERK1/2 phosphorylation would also be interesting to be analyzed in other immune cells like neutrophils or dendritic cells. In addition, C5aRs are also expressed on cells without myeloid origin like endothelial cells, which are also capable of sending danger signals in response to inflammation stimuli like C5a (191). A possible interplay between macrophages, endothelial cells and neutrophils would be very interesting to be studied further, especially in the dependence on the anaphylatoxin receptors. Here it would be very helpful to be able to directly link functions of the ATRs *in vivo* to the different cell types. This could be done using floxed GFP-ATR reporter knock-in mice. In this system, the mating of these mice with LysMCre mice would then specifically knock-out anaphylatoxins in neutrophils and macrophages for example (185).

4.6. The role of C3aR, C5aR1 and C5aR2 in human whole blood

Murine experiments regarding the ATRs yielded very interesting and promising novel insights into the interplay between *Nme*, the complement system and the IMD pathophysiology. In their natural environment, *Nme* depend on the human host (1). In order to translate the roles of C3aR, C5aR1 and C5aR2 as found in the mouse IMD-model to *Nme*'s natural host, a human whole blood system was used. The individual ATRs were blocked with different reagents. These experiments yielded similar effects as seen *ex vivo* in murine PMNs from ATR knock-out mice. The usage of compstatin revealed that the analyzed neutrophil functions all depend on the complement system, as without it and its subsequent release of C3a and C5a all reactions were significantly reduced.

There were differences seen, when C3aR was blocked in human PMNs compared to PMNs from *C3ar*$^{-/-}$ mice. ATRs are expressed on a great variety of cells (74). However, a recent study showed that C3aR is apparently not expressed in murine neutrophils (187), which would explain

why effects were seen in human PMNs when the C3aR was blocked, but not in murine PMNs from *C3ar*$^{-/-}$ mice. This would be explained by the lack of C3aR expression on these murine PMNs. The same would have applied to the use of the superagonist in murine PMNs, which was however only used on human PMNs. There was no change in the oxidative burst, degranulation or phagocytosis upon C3aR activation with the superagonist, but an increase of IL-8 secretion was observed in human PMNs. This could indicate that C3aR is linked to the expression of cytokines like IL-8 in human neutrophils, but if a stronger recruitment of neutrophils would be beneficial in a human setting cannot be concluded from these data. In mice the systemic application of C3aR superagonist did not yield an improvement to the survival, but this might be different in a human setting, in which neutrophils would express the C3aR. On one hand, it could be that an even stronger mobilization of neutrophils might not be beneficial due to a possible increase of damage caused by more neutrophils (188). On the other hand, might the infection also be cleared faster with a high number of neutrophils. The depletion experiments also revealed that neutrophils play an important role in IMD. The phagocytosis was strongly reduced upon C3aR blockade, which could indicate a rather negative effect of C3aR blockade. Phagocytosis is an important defense mechanism in neutrophils (70) and a reduction might have negative consequences. Therefore, it can be postulated that targeting C3aR might not be beneficial in a human setting, as also displayed in the detrimental effects of IMD in *C3ar*$^{-/-}$ knock-out mice.

Lack of C5aR1 showed a reduction in degranulation and oxidative burst in murine PMNs, but no changes were seen when C5aR2 was absent. In human PMNs blocking either C5aR1 or C5aR2 showed a significant reduction in oxidative burst, degranulation and even in the secretion of the neutrophil chemoattractant IL-8. First, this could mean that C5aR2 is functionally linked to different signaling cascades in human PMNs than in mice PMNs. Additionally, the reduction of IL-8 when C5aR1 or C5aR2 was blocked means that less neutrophils would be attracted by activated neutrophils. These findings are in line with IMD experiments *in vivo*, where the lack of one of the three ATRs also reduced the measured amounts of CXCL-1 3 h and 12 h post infection. Interestingly, this would mean that neutrophils might be less recruited by blocking C5aR1 or C5aR2 in mice as well as in humans. In mice, the recruited numbers of neutrophils however would have to be measured to verify a possible change. Other chemokines like MCP-1 were only significantly reduced without C5aR1 expression. Additionally, these experiments could also show that there is a link between the signaling from C5aR1 and C5aR2 and that both, as Hsu *et al.* stated, are working in concert (190).

Regarding phagocytosis, no changes were seen in either murine nor in human PMNs upon blockade or absence of C5aR1 or C5aR2. This stands in contrast to others, who saw a reduction of phagocytosis in human whole blood upon C5a blockade (192, 193). This could be caused by different experimental set ups and differences in the unknown status of donors, that are immunized against *Nme* vs the ones that were not immunized.

Altogether, this work shows that targeting C3aR cannot be recommended for a better outcome of IMD either in mice or humans. Blockade of C5aR1 or C5aR2 however, seems to reduce the immune reactions to a level in mice and humans, which is better for fighting off IMD. Therefore, blocking C5aR1 and C5aR2 could be considered for future therapeutic use in humans, but more research would be needed.

4.7. The role of C3aR, C5aR1 and C5aR2 in murine nasopharyngeal colonization

The complement system does not only play a role in blood, but also in mucosal sites. Complement factors have been detected in bronchioalveolar- (96) as well as in nasopharyngeal lavage (194). Next to the liver, complement factors are also expressed by bone marrow derived cells (195, 196) and cells of non-myeloid origin like epithelial cells (197). Therefore, complement activation has also been seen in mucosal sites, measured via C3a and C5a content upon influenza infections in nasal lavage (198) or in bronchioalveolar lavage in patients with allergic asthma (199). This highlights that the complement system could also have a role on nasopharyngeal colonization by meningococci, which was therefore analyzed by Johswich *et al*. (101). They observed a reduction of colonization, when they strongly activated the complement via CVF shortly before murine i.n. *Nme* infection. These findings suggested that the complement system might be hindering meningococcal nasal colonization (101). For this thesis i.n. infection experiments in huCEACAM-1 expressing mice, lacking additionally either C3, C5, C3aR, C5aR1 or C5aR2, were performed. After certain colonization time points, remaining bacteria were extracted and enumerated to analyze possible differences in the colonization in dependence of the complement system and anaphylatoxin receptors.

Experiments using huCEACAM-1 expressing mice lacking either C3 or C5 did not change the amount of colonization at early or late time points. Additionally, no difference was observed when the anaphylatoxin receptors were knocked-out. These findings might suggest that the complement system and the anaphylatoxins are not directly affecting the mucosal colonization by *Nme*. These findings also come in line with the observation by Johswich *et al.*, that the colonization of mice was not changed, when mice were infected intranasally 30 h post CVF treatment. In their experiments the lack of the whole complement system (101) and here the lack of the individual factors C3 or C5 did not change the outcome of the colonization. Nevertheless, it also has to be mentioned that there were experimental differences between the two studies. The experiments here were done using huCEACAM-1 expressing mice on a B6 background, whereas in the paper from Johswich *et al.* huCEACAM-1 expressing mice on a FVB background were used (101). FVB mice in contrast to B6 mice are known to have a 2-base pair deletion in the *Hc* gene (200, 201), which leads to a C5 deficiency (202) and could thereby have altered the effect of the CVF for example. On the other hand, the strong activation and the subsequent rapid release of C3a or C5a for example could have triggered further cellular mechanism. Mast cell activation (153) and the subsequent vasodilation and vascular

permeabilization by the released histamines (203) or neutrophil recruitment and activation (204) might have led to the reduced colonization observed by Johswich *et al.* (101). These results would only be seen in the special case of CVF application and not be encountered in the absence of C3 or C5. All in all, it can be said that neither the lack of the complement factors C3 or C5 nor the lack of one of the three anaphylatoxin receptors had a positive or negative impact on the mucosal colonization of Mc58 in huCEACAM-1 expressing mice on a B6 background.

4.8. Type II CRISPR/Cas system in murine nasopharyngeal colonization

Sampson *et al.* found a reduced virulence in *Nme* mutants lacking *cas9*, the essential gene of the type II CRISPR/Cas system (119). This led to test the hypothesis, if the type II CRISPR/Cas system of *Nme* affects their *in vivo* fitness in colonizing their host. In collaboration with the group of Prof. Christoph Schoen, different *Nme* mutants, lacking genes of the type II CRISPR/Cas system, were tested in a murine nasal infection model. The competitive indices showed no clear advantage or disadvantage for *Nme*, when the tested type II CRISPR/Cas system genes were present or absent. Additionally, the two mutants that were lacking the *cas9* gene displayed two different results and also the complementary strains for the *cas9* mutants and the *trc* mutant were not congruent with the 8013 WT strain. It has to be mentioned that although most mice were successfully infected, the number of recovered CFUs differed from animal to animal. Nevertheless, the CFU count of most recovered samples were higher than 10 and therefore this does not explain the discrepancies seen in the results. Comparing the experiments from Sampson and colleagues (119) to this study, there was not only a difference in the *Nme* strains used, Sampson and colleagues used the *Nme* strain 92045, which expresses the W-serotype capsule proteins (119), compared to the *Nme* strain 8013 used in this study expressing the C-type capsule proteins (130). The more prominent difference however was the tested tissue and assay. Sampson and colleagues tested the *in vitro Nme* adhesion assays with human A549 adenocarcinoma alveolar epithelial cells (119), which are different to murine nasal epithelial cells in this *in vivo* study and could lead to different results. Simultaneously to this work, Nadja Heidrich and colleagues in collaboration with the group of Prof. Christoph Schoen further tested *Nme* strain 8013 mutants lacking parts of the type II CRISPR/Cas system. For their adhesion assay they used Detroit562 cells, which have a human nasopharyngeal epithelial origin (121). They observed a reduced adhesion of the different mutants and thereby confirmed Sampson and colleagues observations (119, 121). As Heidrich *et al.* used mutants on the same

8013 meningococcal strain background (121), which were also used in this study, a difference in results due to different strains can be ruled out. Another difference between the publications and this study is the dependence on the adhesin Opa in the used huCEACAM-1 murine nasal infection model (101). In this work 8013 Opa positive strains were used, but the 8013 strain normally has the alleles coding for Opa switched off. Switched off clones were used by Heidrich and her colleagues for their adhesion experiments with the CRISPR/Cas mutants and therefore their results of a reduced adhesion were observed independent of Opa expression (121). In addition to the adhesion assays, Heidrich and colleagues further investigated a possible involvement of the type II CRISPR/Cas system in gene expression via transcriptome analysis. Their analysis revealed a possible impact on a basic lipoprotein, labeled as NMV_0031 and a non-coding RNA, labeled NMnc0040. Thereby, they postulated that Cas9 might act indirectly with the biogenesis of the *Nme* type IV pilus, the minor adhesins Nhh, App or NadA or via interference with Blp and could thereby directly change the arrangement of the bacterial cell wall (121). In conclusion, there was no clear trend visible that the type II CRISPR/Cas system is involved in the nasal colonization in the humanized CEACAM-1 expressing murine model, used in this thesis. Furthermore, the lack of the two trans-acting sRNAs RcoF1/2 and thereby a possible increase in the colonization factor PrpB (131), also did not yield an colonization advantage in this infection model compared to the WT strain. Even though unlikely, the recovery rate of CFUs after infection might not have been sensitive enough and thereby could have led to opposing effects in mutant and complemented strains. Finally, it is most likely, as it has been shown by Heidrich and colleagues, that the mechanisms by which the type II CRISPR/Cas system might be involved in the virulence of *Nme* is actually Opa independent or specific for human endothelial cells (121). A possible involvement of the type II CRISPR/Cas system on mucosal colonization of *Nme* could therefore not be observed in the Opa dependent huCEACAM-1 expressing murine nasal infection model.

5. Conclusion

This thesis aimed to analyze the involvement of ATRs in IMD and in mucosal colonization. It was shown that the C5aR1 and the C5aR2 have a detrimental role in IMD, whereas the expression of C3aR was protective. The beneficial effect could be reproduced in WT mice by therapeutically targeting both C5aRs simultaneously. Depletion experiments revealed that next to the complement system also cellular components of the innate immunity are important for a successful *Nme* clearance. Neutrophil analysis showed a milder response, when C5aR1 was not expressed, but no changes were seen when C5aR2 was absent. A common reaction of C5aR1 and C5aR2 was seen in murine bone marrow derived macrophages. The lack of either C5aR showed a different initial response to *Nme*, compared to WT macrophages. In contrast to murine neutrophils, in human neutrophils the blockade of either C3aR, C5aR1 or C5aR2 reduced the oxidative burst, the degranulation and cytokine release and showed a significant reduction in phagocytosis after blocking C3aR. This indicates that C3aR is important for a correct function of neutrophils and that the absence of C5aR1 and C5aR2 might reduce possible damage dealt to the host by overactivated neutrophils. Lack of ATRs, C3 or C5 in mucosal colonization on the other hand had no impact on the colonization of *Nme*. In this huCEACAM-1 colonization model, dependent on bacterial Opa expression, no changes could be observed in the colonization dependent on the type II CRISPR/Cas system or the two trans-acting sRNAs RcoF1/2. In short, ATR play no role in nasopharyngeal colonization, but are very important in IMD in murine and human settings and C5aR1 as well as C5aR2 are possible candidates for future additional treatment options in IMD patients.

6. Summary

The human specific gram-negative bacterium *Neisseria meningitidis* (*Nme*, meningococci) is a common colonizer of the upper respiratory tract. Upon becoming invasive, *Nme* can cause meningitis and life-threatening sepsis. The most important immune defense mechanism in invasive meningococcal disease (IMD) is the complement mediated killing of bacteria. The complement cascade is activated through different pathogen associated patterns and finally leads to the lysis of the bacteria by the membrane attack complex. In addition to the direct bacterial killing, the complement system is also an important player in different inflammatory processes. A hallmark of IMD is an overreaction of the immune system and the release of the potent anaphylatoxins C3a and C5a by the complement system is an important factor hereby. There are three anaphylatoxin receptors (ATRs), the C3aR, the C5aR1 and the C5aR2, capable of detecting these anaphylatoxins. It has already been shown that blocking the ATR C5aR1 strongly benefitted the outcome of IMD in a murine sepsis model. However, the roles of ATRs C3aR and C5aR2 in IMD are still unclear. This work aims to analyze the role of these ATRs in meningococcal sepsis and to identify possible underlying mechanisms. Furthermore, a possible involvement of the complement system, the ATRs and the type II CRISPR/Cas system on nasopharyngeal colonization is analyzed.

In vivo depletion experiments showed that without neutrophils or monocytes/macrophages the complement system alone was not able to clear a low dose *Nme* infection, which highlights the importance of cellular components in IMD. Analyzing the role of the ATRs in knock-out mice with high dose *Nme* infections, revealed that the lack of C5aR2, like the lack of C5aR1, was beneficial for the outcome of meningococcal induced sepsis. In contrast, the lack of C3aR in knock-out mice was detrimental. The positive outcome associated with the C5aRs could be reproduced by using an antagonist against both C5aRs or an antagonist specifically against C5aR1 in WT mice. These findings are giving hope to future therapeutic applications. Next, a possible contribution of neutrophils to this positive outcome was analyzed. Absence of C5aR1 led to a decrease of degranulation by neutrophils in a murine whole blood model, while the other ATRs showed no effect. Neutrophil analysis in human whole blood, on the other hand, revealed a reduced oxidative burst and IL-8 secretion upon inhibition of all three ATRs. A functional difference between the C5aRs and the C3aR in neutrophils was observed in phagocytosis, which was reduced upon C3aR inhibition, but was unaltered with C5aR1 or C5aR2 inhibition. Possible underlying mechanisms in the phosphorylation of ERK1/2 were analyzed in bone marrow derived macrophages isolated from ATR knock-out mice. The later

phosphorylation of ERK1/2 in macrophages without C5aR1 or C5aR2 expression might explain, why blocking the C5aRs is beneficial for the outcome of IMD in mice. In contrast to these findings, the colonization of the nasopharynx in huCEACAM-1 expressing mice by *Nme* did not seem to depend on the Complement system factors C3 and C5 nor the ATRs. Additionally, no difference in the colonization could be observed in this model using *Nme* mutants lacking different parts of the type 2 CRISPR/Cas system.

Conclusively, this work highlights the importance of the complement system, the ATRs and the cellular components in IMD. Contrariwise, these factors did not play a role in the analyzed nasopharyngeal infection model. The beneficial effects of C5aR1 and C5aR2 lack/inhibition in IMD might have medicinal applications, which could support the standard therapies of IMD in the future.

7. Zusammenfassung

Das human spezifische pathogene Gram-negative Bakterium *Neisseria meningitidis* (*Nme*, Meningokokken) ist ein Kommensale angesiedelt im Nasopharynx. Bei invasiver Erkrankung können *Nme* Meningitis oder eine lebensbedrohliche Sepsis verursachen. Die wichtigste Verteidigung des Immunsystems in invasiver Meningokokken-Erkrankung (IMD) ist die Abtötung von Bakterien durch das Komplementsystem. Die Komplementkaskade wird durch verschiedene pathogenassoziierte Muster in Gang gesetzt und resultiert in dem Aufbau des Membranangriffskomplex, welcher die Bakterien schließlich lysiert. Darüber hinaus spielt das Komplementsystem auch eine wichtige Rolle in verschiedenen inflammatorischen Prozessen im Körper. Ein charakteristisches Merkmal von IMD ist eine übermäßige Reaktion des Immunsystems und dabei ist die Freisetzung der Anaphylatoxine C3a und C5a, durch das Komplementsystem, ein wichtiger Faktor. Es gibt drei Anaphylatoxin Rezeptoren (ATR), den C3aR, den C5aR1 und den C5aR2, welche die jeweiligen Anaphylatoxine erkennen. In murinen Modellen wurde bereits gezeigt, dass die Inhibition des C5aR1 einen positiven Einfluss auf den Verlauf von IMD hat. Im Kontrast dazu sind die Rollen der ATRs C3aR und C5aR2 in IMD weiter unklar. Diese Arbeit hat als Ziel, die Rolle der ATRs in Meningokokken induzierter Sepsis zu untersuchen und mögliche zugrundeliegende Mechanismen zu finden. Des Weiteren soll ein möglicher Einfluss des Komplementsystems, der ATRs und des Typ II CRISPR/Cas Systems auf die Kolonisation durch *Nme* im Nasopharynx untersuchen werden.

In vivo Depletions-Versuche zeigten, dass ohne Neutrophile oder Monozyten/Makrophagen das Komplementsystem allein nicht in der Lage war eine *Nme*-Infektion mit einer niedrigen Infektionsdosis zu beseitigen. Dies zeigt die Wichtigkeit von Immunzellen neben dem Komplementsystem in IMD. Experimente mit hohen *Nme*-Dosen in ATR knock-out Mäusen zeigten, dass die fehlende Expression von C5aR2, wie die von C5aR1, sich positiv auf den Ausgang von IMD auswirkte. Im Gegensatz dazu, verschlimmerte das Fehlen des C3aR Rezeptors den Ausgang der IMD. Die positive Wirkung in den C5aR knock-out Mäusen, konnte auch mit der Gabe von einem gegen beide C5aRs oder einem spezifisch gegen C5aR1 gerichteten Antagonisten in WT Mäusen beobachtet werden. Diese Ergebnisse geben Hoffnung auf eine mögliche zukünftige therapeutische Applikation. Als nächstes wurde eine mögliche Beteiligung von Neutrophilen an dem positiven Ausgang von IMD in Abhängigkeit von den ATRs untersucht. Eine fehlende C5aR1 Expression führte zu einer verminderten Degranulation durch Neutrophile in dem verwendeten murinen Vollblutmodel, wohingegen die fehlende Expression der anderen ATRs keinen Effekt zeigte. Im Gegensatz dazu, zeigten Versuche mit

humanem Vollblut einen verminderten Oxidativen Burst sowie eine verminderte Ausschüttung von IL-8 bei der Blockade von allen drei ATRs. Ein Unterschied zwischen den C5aRs und dem C3aR zeigte sich hingegen in der Phagozytose, welche mit C3aR Inhibierung reduziert war, aber unverändert nach der Inhibierung von C5aR1 oder C5aR2 blieb. Mögliche zugrundeliegende Mechanismen in der Phosphorylation von ERK1/2 wurden anschließend in Knochenmark-gereiften Makrophagen von ATR knock-out Mäusen untersucht. Ohne C5aR1 oder C5aR2 Expression wurde eine verzögerte Phosphorylierung von ERK1/2 in den Makrophagen beobachtet, was erklären könnte warum die Blockade von C5aRs den Ausgang von Meningokokken induzierter Sepsis in Mäusen positiv beeinflusst. Im Gegensatz zu diesen Ergebnissen wurde die Kolonisation des Nasopharynx durch *Nme* in huCEACAM-1 exprimierenden Mäusen, weder durch die Komplementfaktoren C3 und C5 noch durch die ATRs beeinflusst. Zusätzlich konnte auch kein Unterschied in der Besiedelung des Nasopharynx durch *Nme*-Mutanten, die verschiedene Mutationen des Typ 2 CRISPR/Cas Systems besaßen, beobachtet werden.

Diese Arbeit zeigt die Wichtigkeit des Komplementsystems, der ATRs und der Immunzellen in IMD. Zusätzlich zeigt diese Arbeit, dass das Komplementsystem und die ATRs jedoch keine Auswirkungen auf die Kolonisation des Nasopharynx in Mäusen haben. Die äußerst positive Auswirkung auf IMD, wenn C5aR1 und C5aR2 nicht gebildet oder blockiert werden, könnte medizinisch von Bedeutung sein und eventuell in der Zukunft die Standarttherapie bei IMD unterstützen.

8. References

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9. List of Figures

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11. List of Abbreviations

APCs	antigen presenting cells
App	adhesion and penetration protein
ATR	anaphylatoxin receptor / Anaphylatoxinrezeptor
BHI	brain heart infusion broth
BLP	bacterial lipoprotein
Cas	CRISPR associated
CD21	complement receptor 2
CD35	Complement receptor 1
CFU	colony forming unit
CLP	cecal ligation and puncture
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CVF	cobra venom factor
DHR123	dihydrorhodamine 123
dsRNA	double-stranded RNA
Fig.	Figure
G-CSF	granulocyte colony stimulating factor
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinases
H. influenza	*Haemophilus influenza*
HmbR	hemoglobin receptor
HpuAB	hemoglobin-haptoglobin recptor
i.p.	intra peritoneal
IL	interleukin
IMD	invasive meningococcal disease / Meningokokken-Erkrankung
IVC	individually ventilated cages
Lbp	Lactoferrin-binding protein
LOS	lipooligosaccharides
MBL	mannose-binding lectin
MFI	mean fluorescence intensity
MPO	myeloperoxidase
MspA	meningococcal serine protease A
NadA	neisserial adhesin A
NETs	neutrophil extracellular traps
NhhA	*Neisseria* hia homologue
Nme, meningococci	*Neisseria meningitidis*
NspA	*Neisseria* surface protein A
PAM	protospacer adjacent motif
PAMP	pathogen-associated molecular patterns
PF	Purpura fulminans
PMN	polymorphonuclear neutrophil granulocyte
Por	porin
PRR	pattern recognition receptors
PSA	polysialic acid
RcoF1/2	RcoF1 and RcoF2
S. pneumoniae	*Streptococcus pneumoniae*
SPF	specific pathogen free
TbpA	transferrin-binding protein A

TbpB	transferrin-binding protein B
TNF	tumor necrosis factor
tracrRNA	*trans*-activating CRISPR RNA
trc	*tracrRNA*
WSF	Waterhouse-Friderichsen syndrome
WT	wild type
Δ*Δrcof1/2*	double deletion mutant Δ*rcof1* and Δ*rcof2*

12. Publications

Muenstermann M, Strobel L, Klos A, Wetsel R A, Woodruff T M, Kohl J, Johswich K O. Distinct roles of the anaphylatoxin receptors C3aR, C5aR1 and C5aR2 in experimental meningococcal infections. Virulence. 2019;10(1):677-694.

Herrmann J B, Muenstermann M, Strobel L, Schubert-Unkmeir A, Woodruff T M, Gray-Owen S D, Klos A, Johswich K O. Complement C5a Receptor 1 Exacerbates the Pathophysiology of N. meningitidis Sepsis and Is a Potential Target for Disease Treatment. MBio. 2018;9(1).

13. Affidavit

I hereby confirm that my thesis entitled "The roles of the anaphylatoxin receptors during invasive disease as well as mucosal colonization caused by *Neisseria meningitidis*" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Die Rolle der Anaphylatoxinrezeptoren während invasiver Infektion sowie mukosaler Kolonisation verursacht durch *Neisseria meningitidis*" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

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15. Curriculum Vitae