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DEDICATION

To my two most persistent teachers.
Who put all their efforts into my education.
Who encouraged me to think.
Who fuelled my curiosity.

To my two pillars of strength.
Who stood firm behind me.
Who backed me up.
Who never trembled.

To my two oldest friends.
Who showed me the Earth from above.
Who showed me the Oceans from below.
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Beyond science.

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ABSTRACT

The Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae* are two major human pathogens that account for substantial morbidity and mortality world-wide. Both bacteria possess multiple mechanisms to circumvent currently available interventions to treat or prevent disease, such as resistance to multiple antibiotics (*S. aureus*), or reduced vaccine-mediated protection due to changes in serotype-distribution (*S. pneumoniae*). A more profound understanding of the underlying mechanisms in host-pathogen interactions is needed to develop novel strategies combating staphylococcal and pneumococcal infections.

The research findings included in this dissertation comprise new insights into the roles of *S. aureus* leukocidins (LukGH, LukSF-PV, LukED, HlgAB, HlgCB) and *S. pneumoniae* neuraminidases (NanA, NanB, NanC) during infections. These bacterial proteins are regarded as important virulence determinants of the respective pathogens, although their molecular effects on the host greatly differ. The staphylococcal leukocidins are directly cytotoxic to host immune cells, whereas pneumococcal neuraminidases are involved in bacterial sugar acquisition, host colonization and implicated in modulating inflammatory responses.

I provide novel findings that demonstrate how different stimuli, both host-derived and of bacterial origin, can affect *S. aureus* leukocidin-mediated cytotoxicity of human polymorphonuclear leukocytes. Neutralizing leukocidins with antibodies constitutes a novel approach to combat *S. aureus* infections. Experimental data included here demonstrate that the combination of two monoclonal antibodies (ASN-1 + ASN-2, the combination called ASN100) that targets all leukocidins and Hla can protect innate and adaptive human immune cells from *S. aureus*-mediated cytotoxicity.

Despite being regarded as important virulence determinants, *S. pneumoniae* neuraminidases NanA, NanB and NanC have not been studied comprehensively in lung infection models, as most research hitherto has focused on NanA. I provide evidence that pneumococcal neuramidases can remove terminal sialic acids from primary human lung tissue *in vitro* and murine lung cells *in vivo*. NanB and NanC can at least partially compensate for the absence of NanA. All three neuraminidases can enhance the interaction of *S. pneumoniae* with human host cells.

A hallmark of pneumococcal pneumonia is excess inflammation of the host organism. Pneumonia is particularly severe in elderly people (> 65 years of age). New data included here show that young compared to aged mice can more efficiently control pneumococcal replication during pneumonia accompanied by a more balanced local inflammatory response.

In conclusion, by investigating different virulence mechanisms that contribute to pathogenesis, this dissertation provides new insights into the multifaceted interactions of *Staphylococcus aureus* and *Streptococcus pneumoniae* with the host's innate defenses.

KURZFASSUNG

Die Gram positiven Bakterien *Staphylococcus aureus* und *Streptococcus pneumoniae* sind zwei wesentliche Humanpathogene, die weltweit für beträchtliche Morbidität und Mortalität verantwortlich sind. Beide Bakterien besitzen eine Vielzahl an Mechanismen, um zurzeit verfügbare Interventionen zur Behandlung oder Vorbeugung von Krankheiten zu umgehen, wie multiple Antibiotikaresistenzen (*S. aureus*), oder reduzierten Impfschutz durch Veränderungen der Serotyp-Verteilungen (*S. pneumoniae*). Ein fundierteres Verständnis der zugrundeliegenden Interaktionsmechanismen zwischen Wirt und Erreger wird benötigt, um neue Strategien zur Bekämpfung von Staphylokokken- und Pneumokokkeninfektionen zu entwickeln.

Die Forschungserkenntnisse dieser Dissertation beinhalten neue Einblicke in die Rollen der *S. aureus* Leukozidine (LukGH, LukSF-PV, LukED, HlgAB, HlgCB) und *S. pneumoniae* Neuraminidasen (NanA, NanB, NanC) während Infektionen. Diese bakteriellen Proteine werden als wichtige Virulenzdeterminanten der jeweiligen Pathogene angesehen, obwohl sich ihre molekularen Effekte auf den Wirt stark unterscheiden. Die Leukozidine der Staphylokokken sind direkt zytotoxisch für Immunzellen des Wirts, wohingegen Neuraminidasen der Streptokokken im bakteriellen Erwerb von Zuckern und der Kolonisierung des Wirts involviert und in der Modulation von Entzündungsantworten impliziert sind.

Ich lege neue Erkenntnisse dar, die demonstrieren, wie verschiedene Stimuli, sowohl vom Wirt stammend, als auch bakteriellen Ursprungs, die Zytotoxizität von *S. aureus* Leukozidinen auf menschlichen polymorphkernigen Leukozyten beeinflussen. Leukozidine mit Antikörpern zu neutralisieren stellt eine neue Herangehensweise dar, um *S. aureus* Infektionen zu bekämpfen. Experimentelle Daten, die hier enthalten sind, demonstrieren, dass die Kombination zweier monoklonaler Antikörpern (ASN-1 + ASN-2, die Kombination genannt ASN100), welche alle Leukozidine und Hla bindet, humane Zellen des angeborenen und adaptiven Immunsystems vor *S. aureus*-vermittelter Zytotoxizität schützen kann.

Obwohl sie als wichtige Virulenzdeterminanten gelten, sind *S. pneumoniae* Neuraminidasen NanA, NanB und NanC noch nicht umfangreich in Lungeninfektions-

Modellen studiert worden, da sich der Großteil der Forschung bis jetzt auf NanA fokussiert hat. Ich lege Beweise vor, die zeigen, dass *S. pneumoniae* Neuraminidasen endständige Sialinsäuren von primärem humanen Lungengewebe *in vitro* und murinen Lungen *in vivo* entfernen können. NanB und NanC können zumindest teilweise das Fehlen von NanA kompensieren. Alle drei Neuraminidasen können die Interaktion von *S. pneumoniae* mit humanen Wirtszellen erhöhen.

Ein Kennzeichen der Lungenentzündung durch Pneumokokken sind exzessive Entzündungsreaktionen des Wirts. Pneumonie ist besonders schwerwiegend in älteren Menschen (> 65 Jahre alt). Neue Daten, die hier enthalten sind, zeigen, dass junge im Vergleich zu alten Mäusen effizienter darin sind, die Replikation von Pneumokokken während der Lungenentzündung zu kontrollieren, was mit einer besser ausgeglichenen lokalen Entzündungsreaktion einhergeht.

Schlußfolgernd bietet diese Dissertation neue Einblicke in die vielseitigen Interaktionen von *Staphylococcus aureus* und *Streptococcus pneumoniae* mit der angeborenen Abwehr des Wirtes, indem verschiedene Virulenzmechanismen erforscht werden, welche zum Krankheitsverlauf beitragen.

PREAMBLE

Background

Since their first description in the 19th century, the Gram-positive bacteria *Staphylococcus aureus* (1880 by Alexander Ogston) and *Streptococcus pneumoniae* (1881 independently by Louis Pasteur and George M. Sternberg) have been of major interest as human pathogens ^{1,2}. Both organisms can cause a range of different diseases, including pneumonia, which is amongst the most common severe manifestations ³. Antibiotics developed in the middle of the 20th century were initially considered as miracle drugs in combatting staphylococcal and pneumococcal infections. However, soon after their introduction, these bacteria developed multiple resistances. Following their broad introduction, antibiotics started to show reduced activity particularly against *S. aureus* ^{4,5}. Additionally, complications during antibiotic treatments of susceptible strains, such as increased toxin expression (*S. aureus*) ⁶⁻⁸ or overshooting inflammation (*S. pneumoniae*) during pneumonia ^{9,10}, pose a substantial challenge to improve disease outcomes.

In the following chapters, aspects of *S. aureus* and *S. pneumoniae* biology relevant to my research project are outlined; detailed descriptions of each pathogen are beyond the scope of this dissertation. The aim here is rather to pinpoint pathogenic mechanisms that are seemingly attractive targets for novel anti-bacterial approaches that can support or replace current antibiotic regimens in the future.

Staphylococcus aureus

Natural reservoir. *Staphylococcus aureus* (Phylum: Firmicutes, Class: Bacilli, Order: Bacillales, Family: Staphylococcaceae, Genus: Staphylococcus) is a frequent asymptomatic colonizer of humans, other mammals (e.g. cattle, domestic animals) and birds. Host niches for colonization include the skin and mucous membranes such as the anterior nares. It is estimated that around one third of the human population is colonized by *S. aureus* ^{5,11}. Certain factors, such as immunosuppression, malnutrition, ethnicity, breaching of mechanical barriers and access to otherwise sterile anatomical sites, favor the shift of *S. aureus* from a commensal towards a pathogenic lifestyle ¹².

Clinical manifestation. Many diseases can be caused by *S. aureus*, for example skin and deep tissue abscesses, endocarditis, gastroenteritis and importantly pneumonia and bacteremia/sepsis, the latter being the deadliest. *S. aureus* is notorious for its ability to cause pneumonia in hospitalized individuals (HAP), patients requiring mechanical ventilation

(VAP), or individuals in health-care environments (HCAP). However, also community-acquired pneumonia (CAP) caused by *S. aureus* frequently occurs ¹².

Disease burden. Despite its recognition as a major human pathogen, estimates of the global *S. aureus* disease burden are currently missing. Available data on *S. aureus* morbidity and mortality are largely derived from studies evaluating MRSA (methicillin-resistant *S. aureus*) epidemiology in Western countries. Annually, an estimated 170.000 MRSA infections occur in the European Union (approximately 5.000 fatalities) ¹³. In the USA more than 80.000 invasive MRSA infections with approximately 11.000 related deaths per year were reported ¹⁴. Importantly, although regional differences between the prevalence of MRSA and MSSA (methicillin sensitive *S. aureus*) exist, MSSA strains remain the major cause of *S. aureus* infections globally ¹². An extensive study including approximately 20.000 patients from countries in Europe, North America and Australia (recruited over a 9-year period) reported overall annual *S. aureus* bloodstream infection rates of 26.1 per 100.000 population. The majority of isolates were MSSA (24.2 per 100.000 population). Case fatality rates for both MSSA and MRSA bacteremia were approximately 20 % ^{15,16}. A large national cohort study in the USA involving ~ 4500 pneumonia patients from 59 different sites identified *S. aureus* in approximately one quarter of all CAP and nearly half of all HAP, HCAP and VAP cases. Among the patients with *S. aureus* infections MRSA compared to MSSA strains were more frequently isolated in HCAP patients, equal frequencies were seen in HAP, but MSSA strains were isolated in nearly two thirds of both CAP and VAP patients ¹⁷.

Prevention & treatment challenges. No vaccines against *S. aureus* are available. Although many attempts have been made over the last few decades, all anti- *S. aureus* vaccine trials have failed. Notably, these candidate vaccines have all focused on targeting a single antigen. Due to the complexity of *S. aureus* pathogenesis, vaccines targeting multiple antigens are presumably required to provide protection ^{18,19}. Resistances to multiple antibiotics in *S. aureus* isolates are constantly increasing (community-acquired methicillin-resistant *S. aureus*, CA-MRSA and hospital acquired methicillin-resistant *S. aureus* HA-MRSA) and are of global concern ²⁰. As noted above, also MSSA strains, are associated with high mortality despite being killed by conventional antibiotics ^{21–23}.

Selected mechanisms to survive inside the host. *S. aureus* employs many mechanisms to interact with the host during infection and to evade immune responses. MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) are cell wall-anchored proteins (e.g. ClfA&B, FnBPA&B, Cna) that mediate adhesion of staphylococci to the host extracellular matrix (e.g. fibronectin, collagen, elastin). They are also involved in

the formation of plasma clots (furuncles, abscesses) during later stages of infection, which is used by *S. aureus* to shield itself from the immune system⁵. The superantigens TSST-1 (toxic shock syndrome toxin-1) and SEs (staphylococcal enterotoxins) as well as ETs (exfoliative toxin) can cause a systemic collapse of the immune system via the induction of cytokine storms, gastrointestinal symptoms (vomiting, diarrhea), or scalded skin syndrome, respectively²⁴. *S. aureus* can avoid opsonophagocytosis via capsule expression²⁵ and Protein A-mediated binding of immunoglobulins via the Fc part²⁶. Moreover, *S. aureus* can secrete CHIPS (Chemotaxis inhibitory protein of staphylococci) and SCIN (Staphylococcal complement inhibitor) to interfere with complement-mediated neutrophil chemotaxis, phagocytosis and oxidative burst²⁷.

Pore-forming toxins. A unique feature of *S. aureus* is the production of a range of beta-barrel pore-forming toxins to destroy epithelial and immune cells. These include Hla (α -hemolysin), and the leukocidins HlgAB, HlgCB (collectively called γ -hemolysins) LukED, LukGH (LukAB) and LukSF-PV^{28,29}. Cell tropism is mediated by specific recognition of ADAM-10 (Hla)³⁰, or chemokine and complement receptors expressed on cells of the myeloid, and/or lymphoid lineage: CXCR1/CXCR2 (HlgAB, LukED), CCR5 (LukED), CCR2 (HlgAB), C5aR and C5L2 (LukSF-PV and HlgCB), CD11b/CD18 (LukGH) and DARC (LukED, HlgAB). Therefore, *S. aureus* selectively kills phagocytes (all leukocidins), lymphocytes (LukED), red blood cells (LukED, HlgAB) and epithelial cells (Hla)^{31–37}.

Other *S. aureus* toxins with overlapping functions include Hlb (β -hemolysin) and phenol-soluble modulins PSM α , PSM β , and PSM γ (δ -toxin). Hlb binds cholesterol found in membranes of many eukaryotic cell types. The gene encoding Hlb is inactivated in many human *S. aureus* isolates by the insertion of a bacteriophage, although disintegration can occur *in vivo*³⁸. PSMs bind to formyl peptide receptor 2 (FPR2) expressed mainly on neutrophils³⁹. Only PSM- α is cytotoxic, whereas the other PSMs seem to be largely involved in modulating chemotaxis⁴⁰.

Importantly, pronounced species-specific differences in susceptibility towards leukocidins exist. For example, murine cells are resistant to LukSF-PV and compared to human cells are much less sensitive to all other leukocidins. Rabbits more closely resemble, but do not mirror humans in this regard, as they are much more susceptible to LukED but more resistant to LukGH^{41,42}. This can be explained by lack of evolutionary conservation (no expression) or amino acid sequence variations of the cognate receptors. Therefore, no ideal animal models exist to determine the true contribution of *S. aureus* cytotoxins during human disease^{29,42}.

Leukocidins are bi-component, β -barrel, pore-forming toxins composed of different monomeric subunits. Protein sequence identities for S-components (LukS, LukE HlgA, HlgC) compared to F- components (LukF, LukD, HlgB) or Hla are ~ 20 -30 %. Within the respective subunit groups, protein sequence identities are ~ 70 -80 %. S-components bind to their target receptors, followed by recruitment of F-components and assembly of an octameric pore of alternating subunits. Together with non-cognate pairing of S-and F-components (e.g. LukE with HlgB) can thus result in 12 different active heterodimers ⁴³. In contrast, LukG and LukH are more divergent (protein sequence identities ~ 30 -40 % compared to other leukocidin subunits). These subunits dimerize in solution prior to octamerization on the cell surface upon binding to the receptor, CD11b (that is part of the Complement Receptor 3 (CR3), alias Mac-1 formed with CD18) ⁴⁴. Hla (protein sequence identities ~ 20 -30 % compared to leukocidin subunits) forms heptameric pores ⁴⁵. Pore formation disrupts membrane integrity leading to cell death. A cornerstone of immunity against *S. aureus* is the containment of bacteria by phagocytes, which in turn are selectively killed by leukocidins ³². To protect these key players and the host epithelium from leukocidin and Hla mediated-killing during *S. aureus* infection, ASN100, a combination of two monoclonal antibodies, was developed and is currently evaluated in a Phase II clinical trial (ClinicalTrials.gov Identifier: NCT02940626).

***S. aureus* summary.** No vaccines are currently available against *S. aureus*, which has become resistant to multiple antibiotics. *S. aureus* employs many different virulence mechanisms to escape immune defences, one of them being selective killing of immune and epithelial cells by pore-forming toxins. While most other Gram-positive pathogens that rely on the production of pore-forming toxin(s) as major pathogenic mechanism, such as *S. pneumoniae*, *C. tetani* or *C. diphtheriae*, release one or two cytotoxins ^{46,47}, *S. aureus* strains can produce up to six. Species-specificity of *S. aureus* pore-forming toxins has potentially underestimated their true contribution to human disease. Improved *in vitro* and *in vivo* models are needed to more closely model human infections and to delineate the molecular mechanisms that contribute to pathogenesis.

Streptococcus pneumoniae

Natural reservoir. In contrast to *Staphylococcus aureus*, *Streptococcus pneumoniae* (the pneumococcus; Phylum: Firmicutes, Class: Bacilli, Order: Lactobacillales, Family: Streptococcaceae, Genus: Streptococcus) is an exclusively human-adapted pathogen. The pneumococcus selectively colonizes the nasopharynx ⁴⁸. Colonization peaks in the first few years of life ($\sim 55\%$ of children at the age of 3 are colonized), which declines with increasing

age and is virtually absent in childless adults. Interestingly, this inversely correlates with *S. aureus* colonization, which increases with age ^{49,50}.

Clinical manifestation. Pneumococcal spread from the nasopharynx to different anatomical sites can cause a range of disease manifestations including sinusitis, bronchitis, otitis media, pneumonia, bacteremia/sepsis and meningitis ⁴⁸. Pneumococcal CAP (community-acquired pneumonia), the most common severe infection, is associated with bacteremia in ~ 25-30 % of patients ⁵¹.

Disease burden. It is estimated that *S. pneumoniae* is responsible for approximately 14.5 million serious infections per year world-wide and according to the WHO accounts for approximately 1.6 million fatalities. The highest morbidity and mortality rates are observed in individuals at the extremes of age (< 5 years and > 65 years) ⁵¹⁻⁵⁵.

Synergy with Influenza. *S. pneumoniae* superinfections can greatly exacerbate other respiratory infections, such as the flu caused by Influenza viruses. This lethal synergism was particularly severe during the Spanish Flu (1918-1919), which claimed more than 50 million lives. Most fatalities were attributed to secondary pneumococcal infections (rather than influenza *per se*) that caused acute bronchopneumonia ⁵⁶. Although other organisms, including *S. aureus*, were also isolated from lungs of deceased patients, *S. pneumoniae* was by far the most common bacterium isolated. Importantly, even in the 21st century, *S. pneumoniae* remains the leading causative agent of bacterial superinfections during seasonal or pandemic influenza and is associated with increased disease severity ⁵⁷⁻⁶⁰.

Prevention & treatment challenges. As opposed to *S. aureus*, nearly 100 different *S. pneumoniae* serotypes have been identified on the basis of the polysaccharide capsule compositions. It has been appreciated for more than one century that antibodies against capsular polysaccharides (CPS) are protective ⁶¹. Vaccines against the most predominant capsular types (based on the prevalence in the USA) have become available. PPSV-23 elicits antibody responses against 23 serotypes but is not recommended in children ≤ 2 years. PCV-7, PCV-10 and PCV-13 (7-, 10-, and 13- valent vaccine formulations, respectively, conjugated to diphtheria toxoid CRM197) can confer protection in all age groups but cover fewer serotypes than PPSV-23 ^{51,62}. Undisputedly, vaccination has led to a substantial reduction in pneumococcal disease globally. However, strains not targeted by the vaccines increasingly replace vaccine-covered strains ⁶³. Vaccination has been reported to increase frequencies of antibiotic resistance among non-vaccine serotypes ⁶⁴. Serotype distribution differs around the world, therefore regional differences in strain coverage and vaccine-mediated protection exist ⁶⁵. Moreover, limited protection by PPSV-23 or conjugate vaccines is conferred against

non-bacteremic pneumococcal pneumonia in the elderly- a population that is particularly vulnerable to *S. pneumoniae* infection⁶⁶⁻⁶⁸. Of note, the rates of invasive pneumococcal disease caused by non-vaccine serotypes were reported to increase following vaccination^{69,70}.

Compared to *S. aureus*, resistance to multiple antibiotics in *S. pneumoniae* is not as prevalent or concerning yet. However, there is accumulating evidence that many *S. pneumoniae* strains have become resistant to commonly used antibiotics a trend that is expected to further increase in the near future^{71,72}. Importantly, mortality rates in highly susceptible individuals, including the elderly, continue to remain high, despite treatment with adequate antibiotics to which pneumococci are still susceptible⁷³.

Selected mechanisms to survive inside the host. The pneumococcus is equipped with an arsenal of virulence factors that contribute to survival in the host and interact with the immune system. The pneumococcal capsule consists of thick layers of polysaccharides to inhibit opsonophagocytosis, complement activity, mucus entrapment, or capture in neutrophil extracellular traps. It is a prerequisite for virulence as virtually all clinical isolates express it and unencapsulated strains show greatly reduced virulence or are avirulent⁷⁴⁻⁷⁷. Capsule expression is generally downregulated during colonization (to allow interaction of subjugant molecules with host structures) and strongly upregulated during invasion (to protect from blood components, such as complement or antibodies)⁷⁸⁻⁸⁰.

The pneumococcal cell wall is a potent stimulator of the immune response and accounts for the strong inflammatory reaction induced by pneumococci^{81,82}. It contains teichoic acids and serves as the anchor for many virulence factors. Examples include Phosphorylcholine (mediates adhesion to PAFr⁸³), PspA (interferes with C3 deposition⁸⁴, sequesters lactoferrin⁸⁵, protects against bactericidal apo-lactoferrin⁸⁶), PspC (mediates adhesion to pIgR⁸⁷, binds secretory component of IgA and factor H⁸⁸ as well as C3⁸⁹), LytA (induces autolysis⁹⁰), PsaA (transports manganese⁹¹) and PsrP (mediates adhesion to keratin-10⁹²). These surface antigens were shown to be effective targets for vaccination in animal studies^{93,94}. Importantly, none of these virulence factors possess toxic activity towards host cells.

Pneumolysin. *S. pneumoniae* produces a single cytotoxin, pneumolysin (Ply), which is expressed in almost all clinical isolates⁹⁵. This is in stark contrast to the ability of *S. aureus* to produce up to six pore-forming toxins simultaneously that specifically target immune and epithelial cells. The release of pneumolysin does not seem to be an active process, but rather occurs during pneumococcal death (induced by antibiotics, or the autolysin LytA). At sublytic concentrations Ply triggers TLR4 dependent inflammatory responses⁹⁶ and facilitates uptake of pneumococcal DNA by host cells, which in turn induces Type-I IFN signalling⁹⁷. At lytic

concentrations on the contrary, it inserts into cholesterol-containing host cell membranes to form oligomeric pores leading to cell death and tissue damage⁹⁵. Although classically viewed as a pore-forming toxin that unspecifically binds cholesterol, binding to a specific host receptor, P2X₇, has been demonstrated recently⁹⁸. Vaccination of mice with recombinant Ply or gene deletion mutation of *ply* reduces pneumococcal virulence^{46,99}. There is a notion that a certain amount of Ply is beneficial to induce early host responses, whereas at later stages it contributes to excess inflammation and tissue damage⁹⁵. Given its multiple roles in pneumococcal pathogenesis Ply is regarded as a main candidate for future protein-based therapies¹⁰⁰. Targeting Ply alone, however, will likely not suffice to combat pneumococcal disease, as *S. pneumoniae* strains secreting non-hemolytic versions of Ply are still capable of successfully infecting the host and causing disease^{101,102}.

Neuraminidases. Neuraminidase A (NanA) is an additional virulence factor for targeting due to its multiple roles in the pneumococcal life-cycle. Two other neuraminidases (sialidases), NanB and NanC exist, although it is largely unknown whether both can perform redundant functions as the major research focus has hitherto been on NanA. All clinical *S. pneumoniae* isolates harbour the genes for one up to all three neuraminidases (reported frequencies for *nanA*, *nanB*, *nanC* are 100, 96, 51 %, respectively)¹⁰³. Sialidases are involved in pneumococcal sugar acquisition from host cells to enable bacterial growth in environments with limited access to otherwise preferred sugars (such as glucose)^{104,105}. NanA can degrade human mucus thereby presumably facilitating access to the underlying epithelium¹⁰⁶. Controversy exists about the involvement of neuraminidases in colonization and the understanding of their importance during pneumonia is limited so far, which is more extensively discussed in **Manuscript I**. A single report has demonstrated that NanA can induce pro-inflammatory responses by disrupting inhibitory Siglec circuits, however, whether the same holds true for NanB and NanC was not assessed¹⁰⁷. All three neuraminidases have been indicated as virulence determinants during haemolytic uremic syndrome by cleaving host serum glycoproteins (such as complement components, fibrinogens and fetuin A)¹⁰⁸. Most recently, vaccination of mice with a mixture of all three recombinant neuraminidases was shown to increase survival rates comparable to vaccination with PPSV-23 in *S. pneumoniae* bloodstream infection models¹⁰⁹. However, human pneumococcal sepsis usually develops from bacterial infection of the lungs followed by bloodstream invasion. Therefore, it is important to understand the involvement of pneumococcal neuraminidases in pneumonia and whether targeting thereof can improve disease outcomes.

***S. pneumoniae* summary.** Although vaccines against *S. pneumoniae* have substantially reduced pneumococcal disease, incomplete serotype-coverage, reduced protection from pneumococcal pneumonia particularly in the elderly and high mortality rates despite the availability of antibiotics, require improved strategies to fight pneumococcal disease and especially pneumonia. Protection from pneumococcal pneumonia likely requires simultaneous targeting of multiple (protein) antigens, pneumolysin being regarded as one of the major virulence factors. Neuraminidases appear as attractive additional targets due to their multiple roles in the pneumococcal life-cycle, however, the current understanding of the involvement of NanA, NanB and NanC during pneumonia is incomplete.

Monoclonal antibodies (mAbs) to combat infectious disease.

Therapeutic potential of mAbs. During the last few decades more than 70 different monoclonal antibodies have been approved for therapeutic use by the US Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA)¹¹⁰. The majority of these mAbs are used to treat cancer or interfere with uncontrolled inflammatory responses. On the contrary, only four mAbs are currently approved for treating infectious diseases: Palivizumab (Synagis®) targets the F-protein of RSV (Respiratory Syncytial Virus), Obiltoxaximab (Anthim®) and Raxibacumab (ABthrax®) target *Bacillus anthracis* toxin (both FDA approved only) and Bezlotoxumab (Zinplava™) targets *Clostridium difficile* toxin B.

Passive vs. active immunization. Passive immunization with mAbs offers several advantages compared to active immunization (vaccination): (i) Generation of an immune response via vaccination takes time, which is appropriate for the prevention of infections when sufficient time is available but not in the short term or during treatment of acute infections. Passive immunisation can provide immediate protection. (ii) Vaccine-induced immune responses require a functional immune system to generate appropriate antibody responses. Therefore, vaccine effectiveness in immunocompromised individuals can be reduced (e.g. immunodeficiency due to HIV infection, immunosuppression by underlying comorbidities, extremes of age). (iii) Several properties of mAbs can be specifically engineered during product development: modifications of the immunoglobulin subclass Fc regions are useful to induce different effector functions and prolong half-life. Antibody affinities to specific epitopes (which are a major determinant of the neutralizing capability) can be greatly enhanced^{111,112}. These aspects are difficult to control during vaccination. Disadvantages of passive compared to active immunization include for example high expenses, the requirement of rapid microbiological diagnosis, and the lack of protection for re-infection.

Passive immunization vs. antibiotic approaches. MAb-based approaches can induce protection upon single dose, while antibiotics require several doses. Half-lives are longer in the former (weeks) compared to the latter (hours). Antibiotics are active against a range of different bacterial phyla by interfering with processes like cell wall synthesis, ribosomal assembly or DNA supercoiling. This can select for resistant survivors, which accumulate because of target gene mutations or acquisition of mobile genetic elements that confer resistance. Moreover, antibiotics disrupt the beneficial microbiome. In contrast, antibodies targeting multiple (secreted) virulence factors, operate in a pathogen-specific manner by aiding host-mediated clearance and no “genetic stress” is imposed on bacteria. Therefore, development of resistances is less likely.

The future of mAbs. Monoclonal antibodies have the potential to overcome several caveats of commonly used antibiotics during bacterial infection. Without dispute, global vaccination programs will remain the mainstay to prevent a multitude of infectious diseases. However, in cases where no vaccines are available (*S. aureus*), where vaccines have limited protection in certain populations (*S. pneumoniae* in the elderly), in acute settings or immunocompromised patients, monoclonal antibodies offer an attractive approach to combat infectious disease ^{113,114}.

Research outline

When I joined Arsanis, ASN100 (a combination of ASN-1 and ASN-2, two monoclonal antibodies (mAbs) that target all *S. aureus* leukocidins and Hla) and an antibody neutralizing *S. pneumoniae* pneumolysin had been generated. Additional *S. pneumoniae* virulence factors were considered for mAb-based targeting and I was part of the team that discovered the anti-neuraminidase antibodies. Recombinant proteins, bacterial strain collections (including a series of *S. aureus* toxin gene deletion mutants) and antibodies were available at Arsanis. Therefore, I could use these reagents as tools to gain a more profound insight into the involvement of staphylococcal leukocidins and pneumococcal neuraminidases during bacterial interaction with the host.

Both *S. aureus* and *S. pneumoniae* are potent inducers of strong inflammatory host responses, a characteristic feature of pneumonia. Several studies have demonstrated that *S. aureus* leukocidins can stimulate inflammatory responses ^{42,115–117}, which led me to investigate how signals relevant to an infectious *in vivo* milieu in turn can affect leukocidin-mediated cytotoxicity (**Publication I**). Since many different immune cells participate in the defence against *S. aureus*, I wanted to determine whether protection of different cells of the

human innate and adaptive immune system can be achieved by ASN-1, ASN-2 or the combination thereof (**Publication II**). The roles of *S. pneumoniae* neuraminidases NanA, NanB and NanC during pneumonia have been less clearly defined compared to *S. aureus* leukocidins. Neuraminidases do not possess cytotoxic activity, however, contradictory findings regarding their involvement in pneumococcal interaction with the host had been published or were not well understood (including pro-inflammatory activity, sugar acquisition from the epithelium *in vivo*, redundancy of NanA, NanB and NanC). Experimental evidence demonstrating that neuraminidases would have activity against primary human lung tissues and could increase pneumococcal interaction with the host was missing. Moreover, it was not clear whether neuraminidases are active in the murine lung during infection and if so, whether mAb-based inhibition could reduce bacterial growth during experimental pneumonia. Therefore, I addressed these questions to increase the understanding of the involvement of neuraminidases in host-pathogen interaction on the one hand and assess their potential as novel candidates for mAb-based targeting during pneumococcal pneumonia on the other hand (**Manuscript I**). Ultimately, as the elderly population is particularly vulnerable to this disease, I investigated whether this could be due to defects in the early recognition of *S. pneumoniae* driven by inappropriate local inflammatory responses (**Manuscript II**).

PUBLICATIONS AND MANUSCRIPTS

Publication I: Janesch P., Rouha H., Weber S., Malafa S., Gross K., Maierhofer B., Badarau A., Visram Z.C., Stulik L., Nagy E. Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by *Staphylococcus aureus* and IL-8. (2017) J. Infect., 74, 473-483.

Publication II: Rouha H., Weber S., Janesch P., Maierhofer B., Gross K., Dolezilkova I., Mirkina I., Visram Z.C., Malafa S., Stulik L., Badarau A., Nagy E., Disarming *Staphylococcus aureus* from destroying human cells by simultaneously neutralizing six cytotoxins with two human monoclonal antibodies (2018) Virulence, 9, 231-247.

Manuscript I: Janesch P., Rouha H., Badarau A., Stulik L., Mirkina I., Caccamo M., Havlicek K., Maierhofer B., Weber S., Groß K., Steinhäuser J., Zerbs M., Varga C., Dolezilkova I., Maier I., Zauner G., Nielson N., Power C.A., Nagy E., Assessing the function of pneumococcal neuraminidases NanA, NanB and NanC in *in vitro* and *in vivo* lung infection models using monoclonal antibodies (2018).

Submitted to Virulence on April 11th, 2018,
currently under revision (status May 18th, 2018).

Manuscript II: Janesch P., Stulik L., Rouha H., Varga C., Steinhäuser J., Zerbs M., Nagy E., Age-related changes in the levels and kinetics of the pulmonary cytokine and chemokine responses to *Streptococcus pneumoniae* in mouse pneumonia models (2018).

Submitted to Infection & Immunity on May 17th, 2018,
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Publication I

**Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by
Staphylococcus aureus and IL-8**

“A mighty flame followeth a tiny spark.”

Dante Alighieri

Background and summary:

Staphylococcus aureus isolates express up to five leukocidins that can kill cells of the human immune system. The cellular tropism is determined by the expression of certain cytokine and/or chemokine receptors, the binding partners of leukocidins²⁹. Human neutrophils are targeted by all leukocidins. As they are the most abundant cells that migrate towards the site of infection and are relatively easy to isolate from peripheral blood in high numbers, they are used in modeling leukocidin-mediated killing mechanisms *in vitro*. Chemokine and complement receptor expression is a dynamic process that responds to different stimuli during infection. We hypothesized that this might affect sensitivities towards *S. aureus* leukocidins.

Several components, such as LPS (used to activate cells), *S. aureus* culture supernatant, *S. aureus* peptidoglycan or *S. aureus* delta toxin, but also human IL-8 increased sensitivity of human PMNs (polymorphonuclear leukocytes) towards LukGH, but not towards LukSF-PV, LukED, HlgAB, or HlgCB. Some of these stimuli in fact inversely affected sensitivity to the other leukocidins. We determined levels of cellular leukocidin surface receptors to see if changes in expression were accompanied by changes in susceptibility. This indeed was the case, as all tested stimuli increased surface density of CD11b (the receptor for LukGH) on PMNs, whereas several led to reduced receptor availability of CXCR1/CXCR2, C5L2 or C5aR. Finally, using different *S. aureus* toxin gene deletion mutants we could demonstrate that LukGH was the most dominant cytotoxin during human PMN infection *in vitro*. Results published in this study have broader applicability by demonstrating that the potencies of bacterial toxins can greatly differ in *in vitro* assays when stimuli relevant during infection are used to more closely resemble the *in vivo* situation.

Personal contributions:

Performing the experiments shown in Figures 2-7, data analysis, manuscript and figure preparation



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Selective sensitization of human neutrophils to LukGH mediated cytotoxicity by *Staphylococcus aureus* and IL-8



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KEYWORDS

Staphylococcus aureus;
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LukGH/LukAB;
CD11b expression

Summary *Objectives:* *Staphylococcus aureus* produces up to five bi-component leukocidins – LukSF-PV, gamma-hemolysins AB and CB, LukGH (LukAB) and LukED – to evade innate immunity by lysing phagocytic cells. Species specificity of these leukocidins limits the relevance of animal models, therefore we assessed their individual contribution using human neutrophils. *Methods:* Human polymorphonuclear leukocytes (PMNs) were activated with stimuli relevant during bacterial infections and sensitivity to recombinant leukocidins was measured in cell-viability assays. Leukocidin receptor expression was quantified by flow cytometry. *Results:* We observed greatly variable sensitivities of different PMN preparations towards LukGH. Activation of PMNs by lipopolysaccharide (LPS) or *S. aureus* culture supernatant (CS) lacking all leukocidins resulted in higher surface expression of CD11b, the LukGH receptor, and greatly enhanced the sensitivity towards LukGH, eliminating the variability observed with unstimulated cells. In contrast, CS induced a decrease in sensitivity of PMNs to the other four leukocidins and reduced surface staining for their cognate receptors (CXCR1, CXCR2, C5aR, C5L2). Delta-toxin and peptidoglycan mimicked the effect of CS. Moreover, IL-8, an important cytokine in neutrophil activation, also selectively increased LukGH sensitivity. Deletion of *lukGH*, but not other leukocidin genes, prevented PMN killing upon infection with USA300 CA-MRSA.

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Conclusion: Inflammatory signals enhance the susceptibility of human PMNs to lysis by LukGH rendering this toxin dominant among the *S. aureus* leukocidins *in vitro*.

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Introduction

Phagocytic cells, especially neutrophils are the cornerstones of host defense against *Staphylococcus aureus*.^{1,2} *S. aureus* has developed multiple mechanisms to avoid its elimination by phagocytic cells. The most direct one is killing of neutrophils, monocytes and macrophages by secreted leukocidins. These cytolytic toxins – the two gamma-hemolysins (HlgAB, HlgCB), the Pantone-Valentine leukocidin (LukSF-PV), LukED and LukGH (LukAB) – belong to the bi-component beta-barrel pore forming toxin family.^{3,4} It is rather enigmatic that *S. aureus* has evolved to employ five different leukocidins and little is known about their individual contribution to human *S. aureus* infections. The *lukSF-PV* gene is carried on prophages in <10% of *S. aureus* isolates, while *lukED* is found in ~70% and *hlgACB* and *lukGH* in almost all clinical isolates.^{3,4} Consequently, correlating gene prevalence with clinical epidemiology has been informative only for LukSF-PV. Therefore, methodological approaches that directly measure cytotoxic potencies associated with the individual leukocidins are needed to assess their contribution to *S. aureus* diseases.

Recently, receptors have been determined for all leukocidins and identified as important immune molecules that play crucial roles in activation and migration of immune cells. LukSF-PV and HlgCB both employ the complement receptors C5aR and C5L2 for cell targeting.^{5,6} LukED and HlgAB both bind to the IL-8 receptors CXCR1 and CXCR2 and additionally target CCR5 or CCR2, respectively.^{6–8} Based on the high amino acid sequence identity among the leukocidins (up to 82%), it is not surprising that some share receptors on their target cells. LukGH is a unique member of the bi-component staphylococcal toxin family with <40% amino acid sequence identity to the other leukocidins. Moreover, unlike the other four leukocidins, it forms a dimer in solution before binding to its target cells.^{9,10} The LukGH receptor was identified to be CD11b, the alpha-subunit of the integrin $\alpha M/\beta 2$, also known as CD11b/CD18 or complement receptor 3 (CR3).¹¹

Although the identification of these leukocidin receptors led to a better understanding of their cellular tropism, species specificity hinders delineation of their roles in pathogenesis *in vivo*.^{3,4,12} Moreover, *in vitro* studies to date have mostly assessed the cytolytic activity of leukocidins towards myeloid cell lines or isolated neutrophils without the context of the inflammatory milieu that is shaped by the interplay between *S. aureus* and the host.

Therefore we employed *in vitro* assays with primary human neutrophils exposed to physiological priming/activating stimuli to assess the effect on leukocidin sensitivity and the relative contribution of the five leukocidins.

Materials and methods

Bacterial strains and culture supernatants (CS)

The USA300 CA-MRSA strain TCH1516 was obtained from ATCC (ATCC® BAA-1717™). Isogenic mutants lacking the *hla*, *lukED*, *lukSF-PV*, *hlgACB*, and *lukGH* genes were generated in the TCH1516 background by homologous recombination as described previously.¹³ Wild type and mutant strains grown in RPMI (Gibco, Thermo Fisher Scientific) supplemented with 1% casamino acids (Amresco) at 37 °C under constant agitation (200 rpm) showed identical growth characteristics based on OD_{600nm} measurements and CFU counting on sheep blood agar plates. CS from cultures grown in RPMI-CAS to stationary phase (OD_{600nm}: ~1.7) were harvested by centrifugation at 5000 × g at 4 °C, followed by filter sterilization of the supernatant using 0.1 µm pore size PVDF syringe filters (Millipore).

PMN isolation and stimulation

PMNs were isolated from heparinized human blood, obtained from healthy volunteers or the Austrian Red Cross. Cells were purified using Percoll Plus (GE Healthcare) gradient centrifugation, as described previously.¹⁰ Purification methods resulted in PMN purity of >95% determined microscopically by Giemsa staining as well as flow cytometric analysis of forward-versus side-scatter properties. Cell viability was >98% based on Trypan blue (Thermo Fisher Scientific) exclusion. Cells at a concentration of 1 × 10⁶/mL were pre-incubated for 1 h at 37 °C, 5% CO₂ with LPS (purified from *Escherichia coli* O111, List Laboratories), *S. aureus* CS, *S. aureus* peptidoglycan (Sigma–Aldrich), *S. aureus* delta-toxin (synthetic peptide from AnaSpec) or human IL-8 (amino acids Ser28–Ser99, BioLegend) at concentrations indicated in the figures prior to use in cell-based toxicity assays or surface staining for leukocidin receptor expression by flow cytometry.

Recombinant leukocidins

Monomers of HlgAB, HlgCB, LukSF-PV and LukED, as well as the co-expressed LukGH dimer (all based on the TCH1516 genome sequences) were recombinantly expressed in *E. coli* as described previously.^{10,13} Briefly, LukS-PV, LukF-PV, HlgA, HlgC and HlgB were expressed in soluble form with an N-terminal NusA/His6 tag, which was removed proteolytically after the first purification step. Purification typically involved three chromatographic steps: 1. IMAC (immobilized metal affinity column); 2. cation exchange or IMAC; and 3. size exclusion chromatography.¹³ LukE and LukD were expressed without tag and purified by cation

exchange (SP-Sepharose FF, 5 mL, GE Healthcare) and gel filtration (Superdex 75 16/60, GE Healthcare). The LukGH complex was generated by co-transformation of *E. coli* with two plasmids containing different antibiotic resistance markers, carrying either the *lukH* or the *lukG* gene. LukG was expressed as a fusion protein with NusA/His6 at the N-terminus to allow metal ion affinity purification of the complex, while LukH was expressed in un-tagged form. The two proteins were co-purified by IMAC from the soluble fraction. The NusA/His6 tag was removed proteolytically with enterokinase giving the un-tagged, mature LukGH complex which was further purified by cation exchange chromatography using the SP FF column. For the cell binding studies, proteins were labeled with the amino reactive reagent Sulfo-NHS-LC biotin (Thermo Scientific), according to the manufacturer's instructions, yielding biotin levels of 1–3 biotin/protein.

The proteins were assayed for purity by SDS-PAGE, monomeric state by size exclusion chromatography (except for LukGH where dynamic light scattering was used), primary structure integrity by mass spectrometry and secondary structure by circular dichroism, and for functionality in *in vitro* toxin potency assays. Endotoxin content was measured for several toxin batches using the chromogenic Limulus Amebocyte Lysate (LAL Chromogenic Endotoxin Quantification kit, Pierce or ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit, Genscript). Values of up to 700 ng LPS per mg toxin for LukGH and between 0 and 250 ng/mg for the other leukocidins were measured. In the experiments where the concentration-dependent effect of LPS was tested, the corresponding LukGH batch contained ~0.001 ng/mL LPS at 1 nM LukGH concentration.

In vitro leukocidin potency assays

PMNs were used for measuring cytotoxicity induced by recombinant leukocidins as described previously.^{10,13} Briefly, 2.5×10^4 cells/well were exposed to equimolar mixtures of S- and F-components for the bi-component toxins LukSF-PV, HlgAB, HlgCB, LukED or the co-expressed LukGH dimer in neutrophil medium (RPMI-1640 with 10% FCS (Sigma–Aldrich), 2 mM L-Glutamine (Thermo Fisher Scientific)), for 4 h at 37 °C, 5% CO₂. Cell viability was measured by determining cellular ATP levels with the Cell Titer-Glo® Luminescent Cell Viability Assay Kit (Promega) according to manufacturer's instructions. Percent cell viability after leukocidin exposure was calculated relative to control cells pre-incubated with the same stimulating agent in the absence of toxin. Data were analyzed by non-linear regression using Prism 6 (Graph Pad). The toxin monomers alone did not affect PMN viability in the concentration range tested.

Flow cytometry based surface staining

PMNs (1×10^5 /well) were incubated with fluorochrome-labeled antibodies (BioLegend, if not stated otherwise) specific for CD11b (clone ICRF44, Brilliant Violet 421), CD18 (clone TS1/18, PE), CXCR1 (clone 8F1/CXCR1, AlexaFluor488), CXCR2 (clone 5E8/CXCR2, AlexaFluor647), C5L2

(clone 1D9-M12, PE), C5aR (clone S5/1, PE) and the corresponding isotype control antibodies in Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific) supplemented with 0.5% BSA (Biomol) and 0.01% NaN₃ for 30 min at 4 °C. Samples were measured with a CytoFLEX flow cytometer (Beckman Coulter) and data analyzed with FCS express version 4.0 (De Novo Software). Results are expressed as relative change in signal intensity compared to unstimulated control cells unless stated otherwise.

For the detection of LukGH binding to PMNs, 1×10^6 cells/well were re-suspended in neutrophil medium with or without 500 ng/mL LPS for 30 min, washed in HBSS + 0.5% BSA and incubated with 27.25 nM biotinylated LukGH for 30 min on ice. After washing in HBSS, cell-bound toxin was detected with Alexa 488-labeled Streptavidin (Molecular Probes) by flow cytometry. Biotinylated LukS-PV was included as a control. Toxin binding is expressed as median fluorescence intensity (MFI).

PMN infection assays with live bacteria

Overnight cultures of *S. aureus* grown in RPMI-CAS were diluted 1:100 and grown to mid-log phase (OD_{600nm}: 0.5) at 37 °C. Bacteria were harvested, washed with PBS to remove secreted toxins, re-suspended in RPMI-1640 with 10% FCS, 2 mM L-glutamine and 10 mM HEPES (Sigma–Aldrich) and added to 2.5×10^4 PMNs/well at different MOIs. Reactions were incubated for 90 min at 37 °C and 5% CO₂, followed by addition of Calcein-AM fluorescent viability dye (eBioscience) at a final concentration of 4 μM for 30 min. PMN viability was quantified using a fluorescence plate reader (λ_{ex} = 485 nm, λ_{em} = 528 nm). Percent viability was calculated relative to mock-treated cells incubated in the same medium (100% viability).

Results

Human PMNs from different donors display highly variable sensitivities towards LukGH

We determined the cytotoxic potencies of the five *S. aureus* leukocidins – LukSF-PV, LukGH, HlgAB, HlgCB and LukED – towards freshly isolated human PMNs over a broad concentration range by measuring cellular ATP levels. All five leukocidins were highly potent with mean EC₅₀ values ranging from ~0.05 to 3 nM (data not shown).

However, we observed a substantial variability in LukGH sensitivity of PMNs isolated from different blood donors, with EC₅₀ values in the ~0.05–25 nM range (individual and cumulative response curves shown in Fig. 1A and B, respectively). Some donors were tested multiple times and their PMNs displayed variable LukGH sensitivities on different days (data not shown). Importantly, PMN susceptibility to other leukocidins did not show this variability. This is exemplified with LukSF-PV that was tested with all the 25 donors and consistently displayed high potencies with EC₅₀ values < 0.5 nM and >95% PMN killing (Fig. 1).

These data suggest that human PMNs from different isolations have greatly different sensitivities to LukGH, a unique observation among *S. aureus* leukocidins.

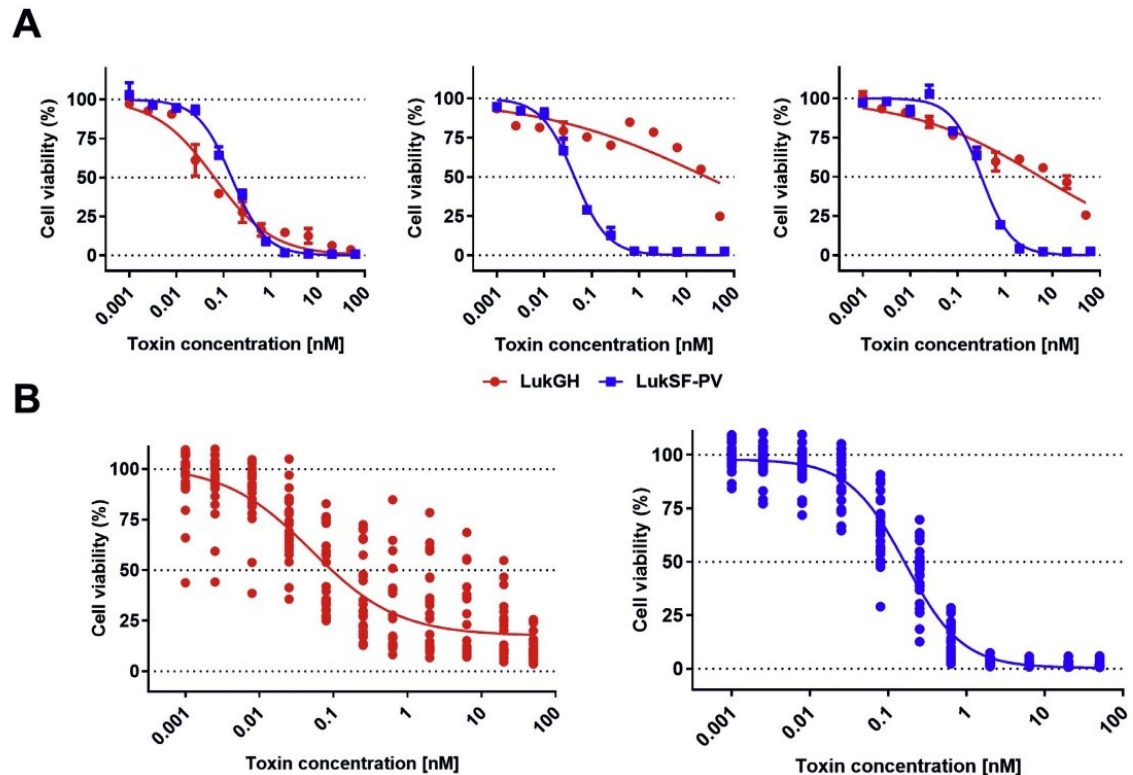


Figure 1 Human PMNs from different donors display variable sensitivity towards LukGH. PMNs freshly isolated from whole blood of healthy adult donors were co-incubated with LukGH or LukSF-PV for 4 h in the indicated concentration range. Susceptibility to these toxins was assessed in a luminescent cell viability assay measuring cellular ATP content. (A) Representative examples from three individual donors, each performed in duplicate. (B) Cumulative dose-response from 25 independent experiments with different PMN preparations and donors. Error bars indicate SEM.

LukGH susceptibility correlates with PMN activation and surface density of CD11b, the LukGH receptor

Neutrophils can be activated by various microbial stimuli for example lipopolysaccharide (LPS) of Gram-negative bacteria, which results in increased surface expression of CD11b that is widely used as an activation marker of

phagocytic cells.^{14,15} Since it also acts as the receptor for LukGH we hypothesized that the different LukGH sensitivities of the human donors may originate from differences in the PMN activation status. This was also supported by our notion that PMNs from the same blood donor can display different LukGH sensitivities when measured on different days.

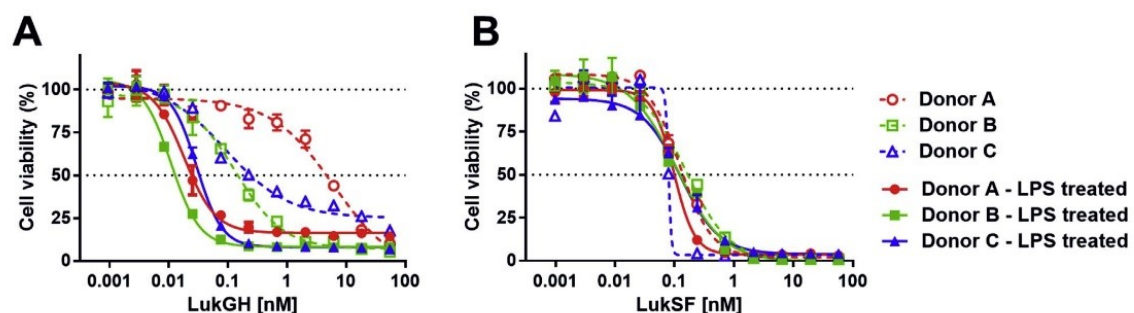


Figure 2 PMN activation via LPS increases sensitivity to LukGH. Purified human PMNs were stimulated with 500 ng/mL LPS for 1 h and then tested for susceptibility towards LukGH (A) and LukSF-PV (B) in the indicated concentration range in a luminescent cell viability assay measuring cellular ATP content. Data represent three independent experiments using different PMN donors, error bars indicate SEM.

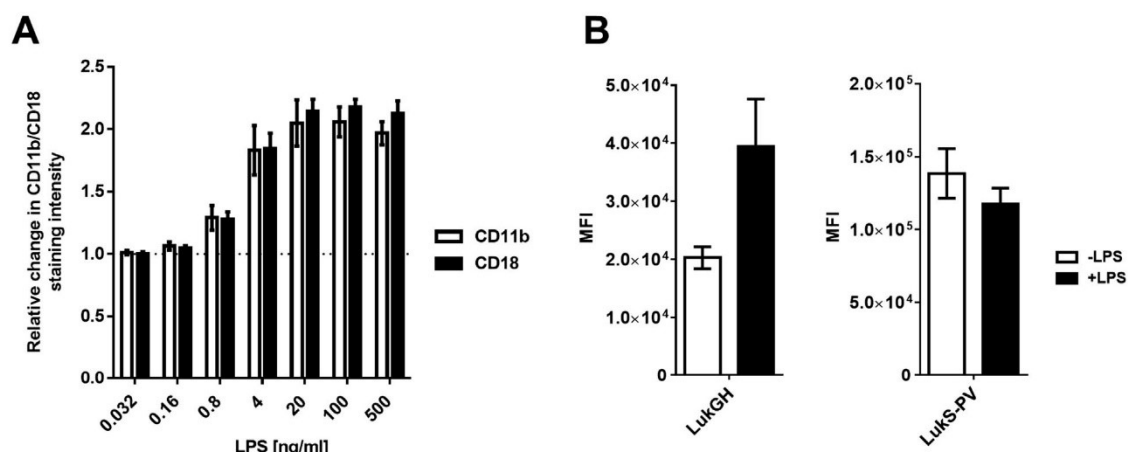


Figure 3 LPS upregulates CD11b/CD18 expression on human PMNs and selectively increases cell binding of LukGH. (A) Purified human PMNs exposed to LPS at the indicated concentrations for 1 h were stained for CD11b and CD18 and expression levels measured by flow cytometry. Results are expressed as relative change compared to untreated cells. (B) Binding of LukGH to cells pre-treated with LPS (500 ng/mL) was measured with biotinylated toxin and a streptavidin-conjugated detection reagent by flow cytometry. Biotinylated LukS-PV was included as a control. Data represent three independent experiments using different PMN donors, error bars indicate SEM.

Therefore, we assessed whether LPS induced activation of PMNs renders them more sensitive to LukGH. In these experiments a substantial increase in LukGH sensitivity was detectable with LPS-treated cells (Fig. 2A) in a concentration dependent manner (Figs. S1A and S2), while susceptibility to LukSF-PV, included as control, did not change (Fig. 2B, Fig. S1B and S2). Interestingly, the high variation in LukGH sensitivity seen with unstimulated cells (EC_{50} values ranging from 0.1 to 6.2 nM) was largely abolished after LPS exposure, as all PMN preparations displayed comparably high sensitivity (EC_{50} values ranging from 0.01 to 0.03 nM) irrespective of their sensitivity without LPS activation (Fig. 2A).

Consistent with the increased LukGH sensitivity upon LPS exposure, we also observed upregulation of CD11b and CD18 on activated cells by flow cytometry (Fig. 3A). This increase was approximately 2-fold, and occurred at higher LPS concentrations than required to increase LukGH sensitivity (decrease in EC_{50} , Fig. S2). LPS-treatment also led to an approximately 2-fold increase in surface binding of biotin-labeled LukGH, not observed with biotinylated LukS-PV, the cell binding entity of LukSF-PV (Fig. 3B).

To delineate how PMN activation by LPS affects the activity of the other leukocidins, we performed further experiments where changes in sensitivity were measured in parallel with receptor surface expression levels for all five

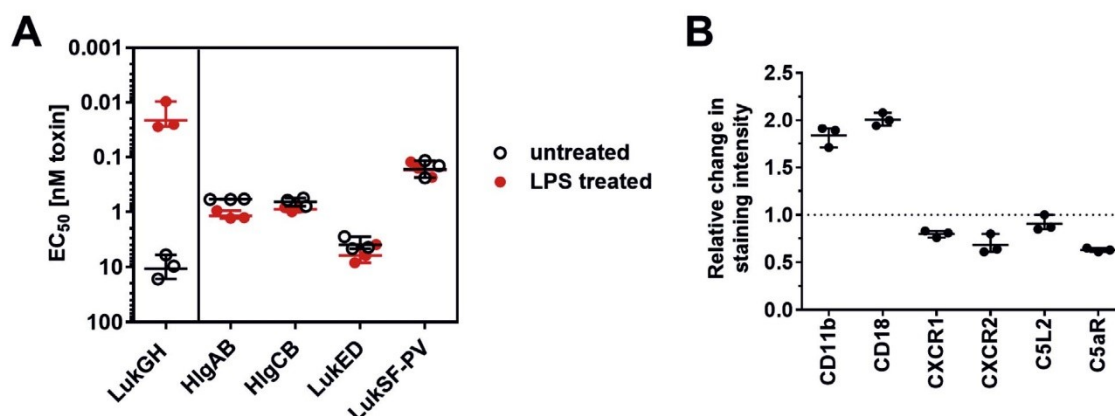


Figure 4 LPS mediated PMN activation increases sensitivity towards LukGH but not to other leukocidins. (A) Human PMNs were pre-treated with 500 ng/mL LPS followed by exposure to individual leukocidins in a 0.005–100 nM dose range. Cell viability was determined after 4 h in an ATP-based viability assay. Potency is shown as nM toxin concentration at the EC_{50} . (B) The effect of LPS (500 ng/mL) on leukocidin receptor staining intensity on human PMNs was assessed with receptor-specific antibodies as indicated. Surface expression levels are expressed as relative change compared to untreated cells. Data are shown as mean + range from three independent experiments using different donors.

leukocidins (Fig. 4 and Fig. S3). These experiments confirmed the increase in LukGH sensitivity (Fig. 4A) and CD11b/CD18 upregulation (Fig. 4B). However, PMN activation did not increase the sensitivity towards HlgAB, HlgCB, LukED and LukSF-PV. This observation was in line with the surface staining results (Fig. 4B and Fig. S3), where no increase in signal intensity was detected for CXCR1/CXCR2 (HlgAB, LukED receptors), C5L2 and C5aR (LukSF-PV and HlgCB receptors).

Taken together these results suggest that PMN activation by LPS results in a selective increase in LukGH sensitivity.

PMNs exposed to *S. aureus* culture supernatant are sensitized to LukGH mediated cytotoxicity and become less sensitive to other leukocidins

S. aureus culture supernatant (CS) was shown previously to induce increased surface density of CD11b on human PMNs.¹⁶ It was therefore tempting to speculate that *S. aureus* itself increases LukGH sensitivity. We tested this by exposing PMNs to CS of a mutant USA300 CA-MRSA strain lacking all leukocidin genes (and *hla*). Although this CS did not affect PMN viability itself, we observed a marked increase in LukGH sensitivity (Fig. 5A). Most importantly, this effect was universal and eliminated differences in susceptibilities of unstimulated PMNs from different donors resulting in similarly low EC₅₀ values (<0.1 nM) as observed upon LPS treatment.

In contrast, upon exposure to *S. aureus* CS, PMNs showed reduced sensitivity towards the other leukocidins with an approximately 10-fold increase in EC₅₀ values (Fig. 5B).

These changes in leukocidin susceptibility of PMNs correlated with the surface staining intensities measured with antibodies against the leukocidin receptors. CD11b expression was increased by up to 2-fold, while C5aR and C5L2 (LukSF-PV and HlgCB receptors), and CXCR1 and CXCR2 (LukED and HlgAB receptors) staining intensities were lower than those obtained with untreated cells (Fig. 5C). The most obvious decrease (~10-fold) was seen for C5aR. Several bacterial components present in *S. aureus* CS have been reported to activate immune cells that results in upregulation of CD11b.^{17–19} We could not confirm such previous findings with *S. aureus* lipoteichoic acid (LTA) or Protein A even at high concentrations (up to 100 µg/mL) and could not detect any changes in susceptibility of PMNs to LukGH (data not shown). Exposure of human PMNs to peptidoglycan and delta-toxin strongly increased sensitivity to LukGH in a concentration-dependent manner (Fig. 6A) but not to LukSF-PV (Fig. 6B). Peptidoglycan at a concentration of 0.2 µg/mL increased the sensitivity to LukGH up to 1000-fold. The delta-toxin effects became obvious above 25 µg/mL. Importantly, we did not observe PMN lysis by delta-toxin itself tested at concentrations up to 100 µg/mL.

Surface staining of PMNs confirmed that the increase in sensitivity to LukGH by peptidoglycan and delta-toxin was paralleled by an upregulation of CD11b, similar to our results obtained with CS and LPS (Fig. 6C).

Collectively, these data confirm that activation of human PMNs by *S. aureus* components selectively increases LukGH mediated cytotoxicity.

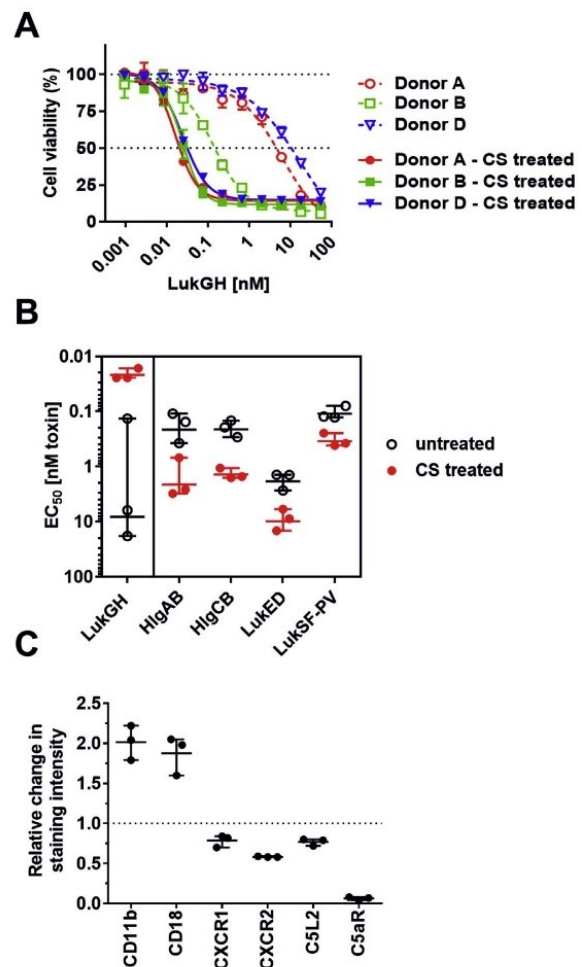


Figure 5 *S. aureus* culture supernatant inversely affects the sensitivity of PMNs to LukGH and the other leukocidins. (A) Human PMNs from three different donors were pre-treated with a 5× diluted, sterile-filtered, non-cytolytic bacterial culture supernatant (CS) of a USA300 CA-MRSA mutant lacking the genes for alpha-hemolysin and all bi-component toxins (TCH1516Δ*hla-lukSF-lukED-hlgABC-lukGH*), followed by exposure to LukGH at the indicated concentration range. Cell viability was determined after 4 h in ATP-based viability assays. Error bars indicate SEM. (B) CS effect on the sensitivity to all other leukocidins was tested in parallel, toxin potency is expressed as nM concentration at the EC₅₀. (C) Effect of CS on leukocidin receptor staining intensity was measured by flow cytometry and expressed as relative change compared to untreated cells. Data in (B) and (C) are shown as mean + range from three independent experiments using different donors.

IL-8 selectively sensitizes human PMNs for LukGH mediated cytotoxicity

IL-8 (CXCL8) is an important chemokine produced by leukocytes, endothelial or epithelial cells in response to pro-inflammatory stimuli that mediates neutrophil activation and transmigration to sites of infection. We therefore

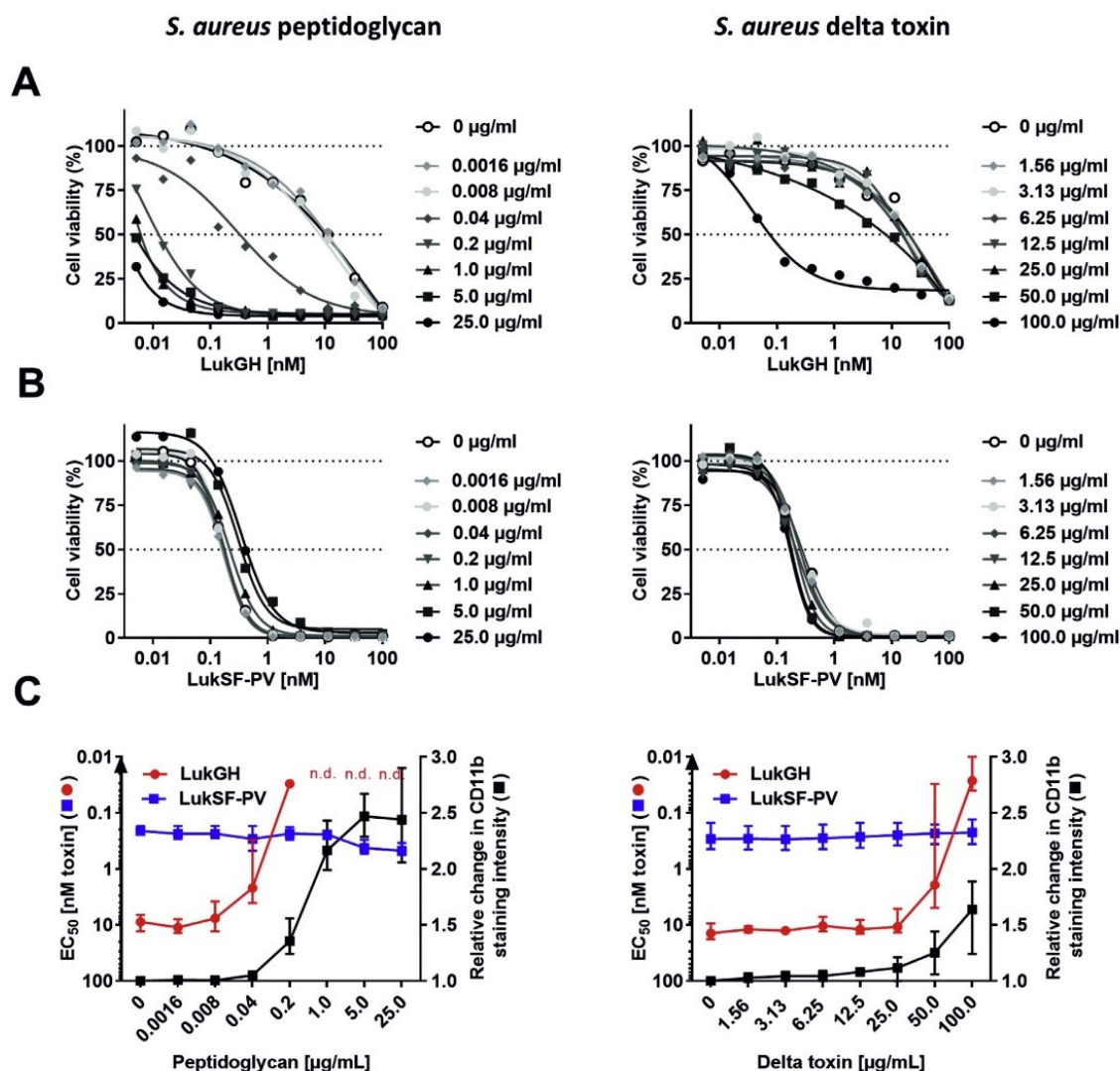


Figure 6 *S. aureus* peptidoglycan and delta-toxin sensitize PMNs to LukGH mediated cell death. PMNs from three donors were exposed to increasing concentrations of *S. aureus* peptidoglycan and delta-toxin, followed by measurement of LukGH and LukSF-PV sensitivity and flow cytometric analysis of CD11b receptor expression. Full titration-curves from ATP viability assays are shown for one representative donor (A, LukGH; B, LukSF-PV); EC₅₀ values for LukGH and LukSF-PV and changes in CD11b surface staining signals from all three donors are summarized in (C); n.d., no EC₅₀ determined (less than 50% viability observed at lowest toxin concentration tested); Data in (C) are shown as mean + range from three independent experiments using different donors.

investigated the potential impact of IL-8 on leukocidin toxicity towards human PMNs. We did not observe changes in LukSF-PV and HlgCB toxicity, but susceptibility towards LukGH greatly increased at low nanomolar IL-8 concentrations (Fig. 7A). Two IL-8 receptors, CXCR1 and CXCR2, have been reported to serve as receptors for LukED and HlgAB.^{6,7} Consistent with these reports, we detected a decrease in LukED and HlgAB toxicity in presence of the cytokine (Fig. 7A). Similarly to what we observed with *S. aureus* CS, CD11b expression increased, while surface binding of antibodies specific for the other leukocidin receptors, especially those detecting CXCR1 and CXCR2, was reduced (Fig. 7B).

These data suggest that IL-8 pre-treatment induces a selective increase in the LukGH susceptibility of human PMNs.

LukGH is the most dominant leukocidin during infection of PMNs with *S. aureus* CA-MRSA USA300

Since the increased toxin sensitivity of activated PMNs was highly selective for LukGH, we subsequently investigated the contribution of the five leukocidins to PMN killing during PMN infection with live bacteria. Human PMNs were infected with wild-type and mutant *S. aureus* USA300 CA-MRSA strains

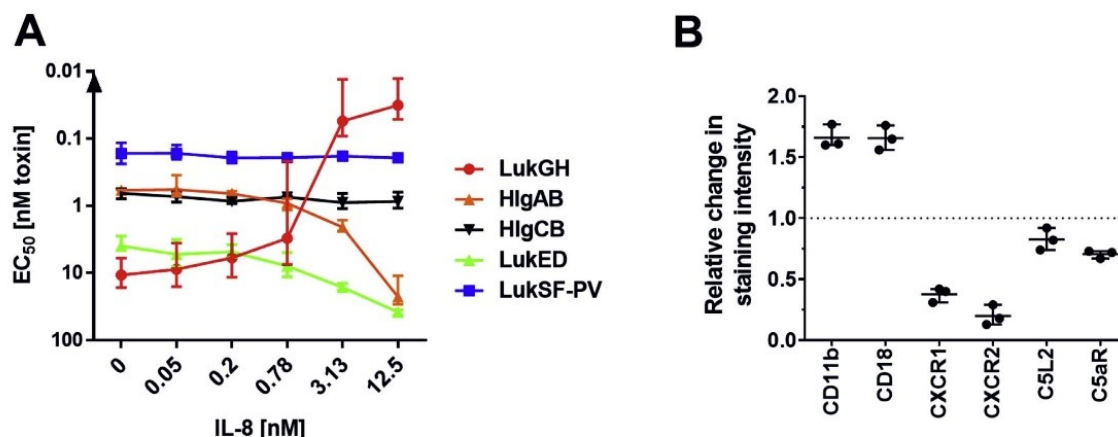


Figure 7 IL-8 changes the sensitivity of human PMNs to the different leukocidins. PMNs from 3 donors were exposed to increasing concentrations of IL-8 for 1 h and then tested for toxin mediated cell lysis with all five leukocidins. (A) Toxin sensitivities, summarized as EC₅₀ values for all tested IL-8 concentrations. (B) Relative change in leukocidin receptor staining intensity upon PMN exposure to 12.5 nM IL-8. Data are shown as mean + range from three independent experiments using different donors.

lacking one or more leukocidin genes at different multiplicities of infection (MOIs) where PMN survival was monitored using a Calcein-AM viability dye. Infection with the wild-type strain at MOI100 and MOI50 resulted in ~75% and 60% killing of PMNs, respectively, while only ~10% of cell death occurred with the mutant strain lacking all leukocidin genes (and *hla*) suggesting that PMN toxicity measured in this assay is mainly driven by these cytotoxins. Infection at MOI10 had very little impact on PMN survival (Fig. 8).

To assess the contribution of the individual leukocidins, we tested a total of 8 mutants that lacked or retained only one of the five leukocidin genes. At the higher MOIs that substantially reduced PMN viability, it was mainly the presence of *lukGH* that influenced cell viability with little contribution of the other leukocidin genes (Fig. 8B and C).

Based on these data, LukGH has the greatest impact on PMN toxicity under conditions where leukocidins are produced *in situ* and live *S. aureus* interacts directly with neutrophils.

Discussion

All five leukocidins produced by *S. aureus* target phagocytic cells, mainly neutrophils by specifically binding to important immune receptors that are under a complex regulation during inflammatory conditions such as bacterial infection. Therefore we studied the effects of different *S. aureus* derived stimuli on leukocidin sensitivity of human PMNs in parallel with cognate receptor expression.

Freshly isolated neutrophils from healthy human donors displayed variable sensitivities to LukGH. This phenomenon was mentioned by others without showing experimental data.²⁰ Importantly such variation in PMN sensitivity was not seen with the other four leukocidins, exemplified by LukSF-PV. We hypothesized that this might be due to variable activation state of PMNs. CD11b, the receptor for LukGH,¹¹ has multiple roles (complement binding, phagocytosis, adhesion and migration) and is an activation marker

for phagocytic cells.^{21,22} CD11b upregulation occurs under inflammatory conditions as a result of neutrophil priming and activation, a process that is inducible by both pathogen-derived molecules such as LPS and host cell-derived factors such as pro-inflammatory cytokines.^{23,24} Therefore, we exposed PMNs to LPS and indeed detected up to 1000-fold increased sensitivity to LukGH and also increased surface levels of CD11b up to 2.5-fold. We found that LukGH binding to neutrophils increased proportionally to CD11b levels (~2.5 fold), while LukS-PV binding remained unchanged, which is consistent with the selectivity of LPS on leukocidin sensitivity. Changes in PMN sensitivity to LukGH-mediated lysis were detectable at lower LPS concentrations than the increase in CD11b staining. This observation can be explained by earlier studies showing that activation of neutrophils does not only result in quantitative changes in CD11b surface expression levels, but also affects its cellular distribution and surface localization pattern (even distribution on resting PMNs, while patchy appearance on activated cells), and conformational state that can happen before recruitment of CD11b from storage granules and *de novo* protein synthesis.^{15,25} Thus, the increased receptor densities in micro-domains on the cell surface might be responsible for the prominent increase in LukGH sensitivity despite the modest increase in total CD11b staining.

Since LPS is derived from Gram negative bacteria, we used it as a tool for PMN activation and modulation of CD11b expression. The *S. aureus* relevant aspects of PMN activation were tested by exposure to cell-free *S. aureus* culture supernatant (CS). It was reported that *S. aureus* CS activates PMNs and results in increased CD11b/CD18 expression.¹⁶ We confirmed these findings with a CS lacking all leukocidins (and *Hla*) and detected a prominent increase in LukGH sensitivity of PMNs, comparable to that induced by LPS. An important finding of this study is the reciprocal effect of *S. aureus* CS on the susceptibility of PMNs to the other leukocidins that was accompanied by decreased surface staining signals for their cognate receptors. Veldkamp

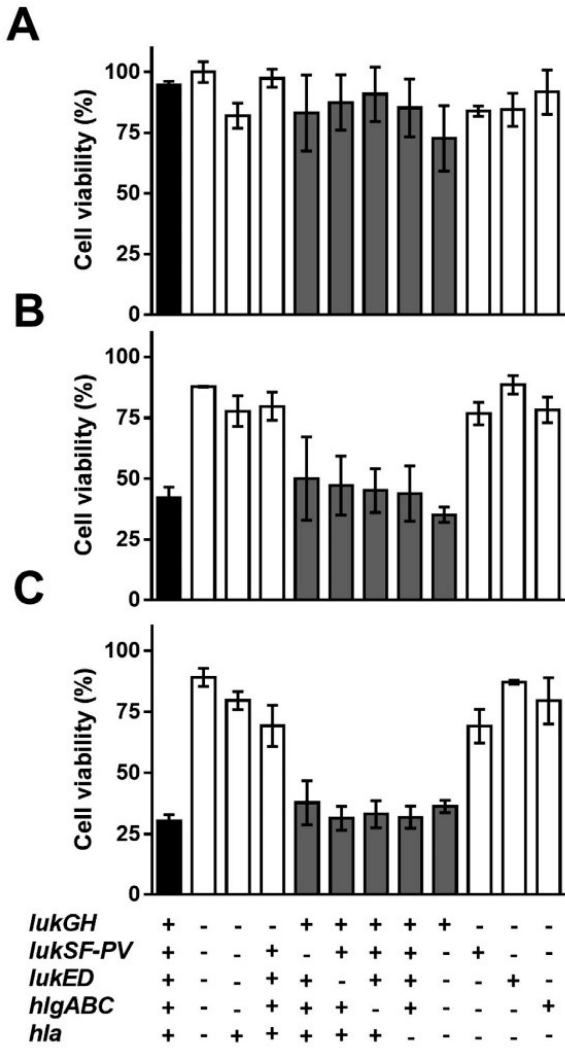


Figure 8 LukGH is the dominant cytotoxin during PMN infection by *S. aureus*. Purified human PMNs were infected with a USA300 CA-MRSA wild-type strain (black bars) and isogenic toxin gene deletion mutants carrying (grey bars) or lacking (white bars) the gene for LukGH at MOI 10 (A), 50 (B) and 100 (C) for 2 h. PMN viability was measured with a Calcein-AM viability dye and is expressed as percentage relative to non-infected cells. Data are presented as mean $\pm$ SEM of two independent experiments with different donors.

et al. also reported that treatment of human neutrophils with *S. aureus* CS resulted in decreased surface staining for the C5a receptor²⁶ now known to bind LukSF-PV and HlgCB. This might be explained by the presence of certain proteins in the CS such as chemotaxis inhibitory protein (CHIPS), which competes with C5a and LukS-PV for C5aR binding^{5,27} or by Staphopain A, a secreted proteolytic enzyme reported to cleave the N-terminal domain of CXCR2, that serves as the binding site for HlgAB and LukED.^{6,28}

Several *S. aureus* components present in the culture supernatant were shown to activate phagocytes and upregulate CD11b. We could not confirm these previously reported effects of Protein A and LTA shown with mouse monocytes¹⁷ on human PMNs, and we did not observe any change in LukGH sensitivity. A human challenge study demonstrated that LPS, but not LTA induced upregulation of CD11b on neutrophils after instillation of these agents into the lung.¹⁸ The increase in CD11b expression on human PMNs following exposure to peptidoglycan and delta-toxin reported previously,^{16,19} could be reproduced under our experimental conditions and was accompanied by greatly increased susceptibility to LukGH. The effective peptidoglycan concentration range was similar to that reported earlier to increase CD11b expression on human PMNs ($\sim 1\text{--}3\text{ }\mu\text{g/mL}$).²⁴ However, it cannot be excluded that purified *S. aureus* peptidoglycan also contains other PMN activating agents, such as lipopeptides shown to exert strong pro-inflammatory signals via TLR-2.²⁹ Delta-toxin is a phenol-soluble modulin with complex roles in *S. aureus* virulence.³⁰ The delta-toxin concentration we found to effectively increase CD11b expression was higher than previously published,¹⁹ but was still below the cell lytic concentration. Since we used a synthetic peptide, confounding effects of contaminations can be excluded. It has been estimated that *S. aureus* CS from stationary cultures contains $\sim 1\text{ }\mu\text{g/mL}$ peptidoglycan and $\sim 1.4\text{ mg/mL}$ delta-toxin.^{31,32} The concentration of peptidoglycan was shown to increase up to $\sim 50\text{ }\mu\text{g/mL}$ in supernatants of bacteria treated with various beta-lactam antibiotics.³³ Therefore the concentrations we found effective in our *in vitro* model seem to be relevant.

Notably, we also observed an increased sensitivity of PMNs to LukGH upon exposure to the pro-inflammatory cytokine IL-8. This key neutrophil chemoattractant and activator is produced rapidly after the onset of infection by neutrophils or epithelial cells,³⁴ for example following exposure to *S. aureus* alpha-hemolysin.³⁵ IL-8 release in response to *S. aureus* peptidoglycan, delta-toxin and LPS, but not LTA has been reported before.^{30,36,37} IL-8 is known to increase the CD11b expression on the surface of neutrophils and to upregulate phagocytic functions with very fast kinetics involving membrane translocation from storage granules.^{38–41} We found that exposure to low nanomolar concentrations of IL-8 increased CD11b on PMNs and was highly effective in increasing LukGH sensitivity. It is also possible that the increased cytotoxicity of LukGH in response to the bacterial factors is compounded by the host response via IL-8 secretion, cellular activation and CD11b upregulation. LukSF-PV and HlgCB sensitivity of IL-8 treated PMNs did not differ from untreated PMNs, while IL-8 markedly reduced HlgAB and LukED mediated toxicity. This could be either due to receptor internalization upon endogenous IL-8 binding or direct competition with the cytokine as demonstrated earlier.^{6,7} Autocrine IL-8 production induced by leukocidin-free CS might account for the reduced CXCR1 and CXCR2 signals observed by flow cytometry.

The contribution of individual leukocidins was also assessed using gene deletion mutants of an USA300 CA-MRSA strain. This clonal lineage carries the genes for all five leukocidins (i.e. *hlgACB*, *lukGH*, *lukSF-PV* and *lukED*). The

effect of *lukGH* deletion on PMN viability was comparable to that observed upon removal of all leukocidin genes, while *S. aureus* strains expressing only LukGH or LukGH in any combination with other leukocidins induced killing comparable to the wild-type strain. Similar data were reported earlier using differentiated HL-60 cells and the *S. aureus* Newman strain that does not carry the *lukSF-PV* gene.⁴² It is difficult to quantify the amount of leukocidins produced *in situ*, as they quickly adsorb onto their target cells. Malachowa et al. reported massively increased leukocidin gene expression of USA300 CA-MRSA, especially those for the gamma-hemolysins upon exposure to humans PMNs.⁴³ Since toxin expression patterns can vary substantially among different *S. aureus* isolates, it will be important to expand this type of analysis and test additional strains.

The relevance of the *in vitro* data obtained with human PMNs is important for the understanding of the true contribution of LukGH to *S. aureus* pathogenesis, as optimal animal models are currently not available. LukGH, similarly to the other leukocidins, displays a marked species specificity, as mice and rabbits have very low to moderate sensitivity to this toxin.⁴⁴

In a broader context, our study also highlights the importance of testing bacterial virulence factors in an *in vitro* setting that more closely mimics the situation *in vivo*. Data generated with recombinant virulence factors and unstimulated cells do not necessarily reflect their real contribution in an inflammatory milieu. The potency differences observed with LukSF-PV and LukGH towards unstimulated versus activated cells serves as a good example. While both leukocidins have comparable activity with unstimulated cells, cell activation clearly renders LukGH the more potent cytotoxin.

Our findings are of importance in the light of development of novel experimental human therapeutics targeting *S. aureus* cytotoxins.^{44–47} Given the dominance of LukGH in the infectious context, targeting this potent toxin is highly warranted.

Funding information

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Conflict of interest

The authors declare a potential conflict of interest as the work was performed at Arsanis Biosciences GmbH (Vienna, Austria), a biotechnology company developing a monoclonal antibody based product targeting *S. aureus* infections.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jinf.2017.02.004>.

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Publication II

Disarming *Staphylococcus aureus* from destroying human cells by simultaneously neutralizing six cytotoxins with two human monoclonal antibodies

“Still round the corner there may wait, a new road or a secret gate.”

John Ronald Reuel Tolkien

Background and summary:

Two previously published studies by Arsanis described the discovery of two monoclonal antibodies, ASN-1 and ASN-2, that potently target *Staphylococcus aureus* leukocidins and Hla^{43,118}. Both the innate and adaptive arm of the immune system contribute to host control of *S. aureus*. By leukocidin- and Hla-mediated killing of resident and recruited cells, *S. aureus* subverts these defenses.

As we had demonstrated before that killing of human PMNs by leukocidins can be greatly influenced by infection-relevant stimuli, this study further evaluated the strain- and growth condition-dependent differences in leukocidin expression levels and cytotoxicity profiles *in vitro*. My task in this work was to determine whether blocking of leukocidins and Hla with ASN-1 and/or ASN-2 could protect cells of the human innate and adaptive immune system from *S. aureus* mediated killing. The results demonstrate that full protection of human granulocytes, monocytes, T cells, B cells and NK cells required simultaneous targeting of all leukocidins and Hla by ASN100 (ASN-1 + ASN-2).

Personal contributions:

Performing the experiment shown in Figure 6, data analysis, assistance during manuscript preparation



Disarming *Staphylococcus aureus* from destroying human cells by simultaneously neutralizing six cytotoxins with two human monoclonal antibodies

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RESEARCH PAPER



Disarming *Staphylococcus aureus* from destroying human cells by simultaneously neutralizing six cytotoxins with two human monoclonal antibodies

Harald Rouha, Susanne Weber, Philipp Janesch, Barbara Maierhofer, Karin Gross, Ivana Dolezilkova, Irina Mirkina, Zehra C. Visram, Stefan Malafa[†], Lukas Stulik, Adriana Badarau, and Eszter Nagy*

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ABSTRACT

Pathogenesis of *Staphylococcus aureus* is increasingly recognized to be driven by powerful toxins. *Staphylococcus aureus* employs up to six pore-forming toxins to subvert the human host defense and to promote bacterial invasion: alpha-hemolysin that disrupts epithelial and endothelial barriers and five leukocidins that lyse phagocytes involved in bacterial clearance. Previously, we described two human monoclonal antibodies (mAbs), ASN-1 that neutralizes alpha-hemolysin and four leukocidins (LukSF-PV, LukED, HlgAB, HlgCB), and ASN-2 that inactivates the 5th leukocidin, LukGH. In this study we tested the individual and combined effects of ASN-1 and ASN-2 in multiple *in vitro* models employing relevant human target cells. We found that diverse *S. aureus* isolates with different genetic backgrounds (based on MLST- and *spa*-typing) and antibiotic sensitivity (both MRSA and MSSA) displayed greatly different cytotoxin expression patterns influenced by the type of growth medium used. Both mAbs were required to fully prevent the lysis of human neutrophils exposed to the mixture of recombinant cytotoxins or native toxins present in the culture supernatants of *S. aureus* isolates. Flow cytometry confirmed the protective effects of ASN-1 + ASN-2 (known as ASN100) on granulocytes, monocytes, NK-cells and T-lymphocytes. ASN-1 alone preserved the integrity of a 3D-primary culture of human tracheal/bronchial mucociliary epithelial tissue infected with *S. aureus*. We conclude that simultaneous inhibition of alpha-hemolysin and five leukocidins by ASN100 blocks cytolytic activity of *S. aureus* towards human target cells *in vitro*.

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monoclonal antibodies; host
defense

Introduction

Staphylococcus aureus is a major human pathogen associated with a significant global health care burden. Approximately 30% of the human population is colonized. However, *S. aureus* can change from a commensal to a pathogen, causing severe disease, such as pneumonia, blood stream infections, osteomyelitis, and complicated skin and deep tissue infections.^{1,2} Methicillin-resistant *S. aureus* (MRSA) has become a global problem and is responsible for life-threatening infections even in young and healthy individuals outside of hospital settings. Despite the availability of appropriate antibiotics, severe MRSA and MSSA infections remain associated with high mortality.¹

The primary innate defense mechanism against *S. aureus* is the control of bacterial growth by phagocytic uptake and bacterial killing, predominantly by neutrophilic granulocytes.³ Antibodies binding to the bacterial surface and activating the complement system greatly

enhance this process (opsonization). Frequent exposure to *S. aureus* results in increasing anti-staphylococcal antibody titers during childhood, and human sera exhibit opsonophagocytic activity *in vitro*.⁴ Despite this, *S. aureus* infections occur repeatedly. All anti-staphylococcal vaccine and passive immunization approaches tested to date in pivotal clinical trials have failed. They have all targeted a single surface structure (i.e. protein, carbohydrate capsule, or LTA) and aimed at opsonophagocytic killing (OPK) as the mode of action. The supportive pre-clinical data, including efficacy in rodent models and *in vitro* OPK activity, generated with these product candidates raises concerns about the relevance of the animal models applied and OPK as a main mechanism of anti-*S. aureus* antibodies.^{5,6}

One of the explanations for the lack of efficacy of antibodies binding to the bacterial surface is the numerous secreted toxins that are produced by *S. aureus* to target host cells for counteracting phagocytosis and to disrupt

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tissue integrity. Uniquely among bacterial pathogens, *S. aureus* secretes up to six different beta-barrel pore forming cytotoxins.⁷⁻⁹ Alpha-hemolysin (Hla) is the best characterized *S. aureus* virulence factor with proven contribution to pathogenesis in all animal models tested.¹⁰ It lyses epithelial and endothelial cells, and is also toxic to lymphocytes and monocytes.¹⁰ The other five pore forming cytotoxins – Leukocidin SF-PV (Panton-Valentine leukocidin), ED and GH (the latter also called LukAB) and the two gamma-hemolysins (HlgAB and HlgCB) – all target leukocytes, and primarily attack phagocytic cells, such as granulocytes, macrophages, and monocytes.¹¹ In addition, LukED lyses lymphocytes and displays hemolytic activity similar to the gamma-hemolysins.^{8,9,11} Hla and LukGH have also been shown to contribute to biofilm production based on gene deletion strains and studies with neutralizing antibodies.^{12,13}

The *S. aureus* leukocidins are highly adapted to the human host and do not elicit appreciable toxicity towards rodent cells with the exception of LukED.^{14,15} Since mice and rats are the most commonly used species for *S. aureus* disease models, the important role of the leukocidins in pathogenesis has been recognized only recently. The lack of phenotype of *lukSF-PV* deletion mutant strains of *S. aureus* in mouse and non-human primate models is explained now by the resistance of phagocytes of these species towards LukSF-PV.¹⁶ Since rabbit phagocytic cells are susceptible to the cytotoxic effect of all *S. aureus* leukocidins, the rabbit represents a more relevant species than the mouse. The prominent role of LukSF-PV in pneumonia pathogenesis was proven in rabbits.¹⁷ Based on *in vitro* assays, the sensitivity of rabbit PMNs for LukSF-PV and gamma hemolysin is comparable to that of human neutrophils, however, LukED is approximately 100-fold more potent and LukGH is 100-fold less toxic to rabbits cells.^{14,15,18}

Species specificity of the *S. aureus* cytotoxins became understood at the molecular level following the identification of their cellular receptors that all belong to chemokine (CXCR1, CXCR2, CCR2, CCR5, DARC) and complement receptor families (C5aR, C3R/CD11b).^{11,19-24} These receptors are either not expressed or not sufficiently conserved in rodents. ADAM10, the receptor for Hla is expressed on human and animal epithelial and endothelial cells, as well as on red blood cells of species susceptible to hemolysis by Hla.²⁵

Dissecting the role of these cytotoxins in human pathogenesis has begun, but as there are little data regarding *in vivo* expression and correlation with disease severity, more efforts and complex approaches are needed. The relevance of LukSF-PV was initially indicated by gene prevalence studies demonstrating the presence of *lukSF-PV* in highly successful CA-

MRSA strains causing severe *S. aureus* infections, such as necrotizing pneumonia and community associated MRSA infections in otherwise healthy people.^{26,27} These observations triggered interest in the other leukocidins. Since the genes encoding Hla, HlgAB, HlgCB, and LukGH are part of the core genome of *S. aureus*, and *lukED* is present in more than 50% of strains, gene prevalence studies provide limited information regarding their role in pathogenesis. Hla expression levels of MSSA isolates have been shown to be associated with the ability to cause pneumonia in mechanically ventilated patients.²⁸ Anti-Hla antibodies were shown to be protective in mouse models of pneumonia, bacteremia, and skin infections,²⁹⁻³¹ and suggested to be protective in humans as well based on the inverse correlation between serum levels and severe *S. aureus* disease in children and adults.^{32,33} It seems that natural infection in general does not provoke durable immunity, since patients can experience recurrent *S. aureus* infections.⁶ Nevertheless, anti-Hla and anti-LukSF-PV antibody levels were shown to inversely correlate with severity in necrotizing pneumonia.^{33,34}

Stimulated by the mounting evidence supporting the important role of the leukocidins in *S. aureus* infections, we have developed human monoclonal antibodies (mAbs) to neutralize Hla and the five leukocidins. A unique type of cytotoxin cross-reactive antibodies that inactivate four of the five leukocidins, HlgAB, HlgCB, LukED and LukSF-PV, and Hla was discovered and one of these mAbs, Hla-F#5 was selected as a development lead, and termed ASN-1.³⁵ ASN-1 recognizes a conserved structural epitope in Hla, HlgB, LukF-PV, and LukD with high affinity.³⁵ ASN-1 was shown to be fully protective in a lethal CA-MRSA USA300 necrotizing pneumonia model in rabbits, while monospecific Hla mAbs tested in the same model were only partially efficacious.^{15,36} Since the binding epitope of ASN-1 is not conserved in LukGH, an additional mAb, targeting this toxin was also developed. Based on its high binding affinity and potent neutralization of the different sequence variants of LukGH, the α LukGH-mAb#5.H1H2 was selected as development lead, named ASN-2.³⁷ The combination of ASN-1 and ASN-2, called ASN100, is currently being tested in a Phase 2 clinical trial for the prevention of *S. aureus* pneumonia in mechanically ventilated patients (NCT02940626).

Due to species specificity, *in vitro* model systems employing human target cells are necessary to assess the individual and collective contribution of the leukocidins to phagocyte damage. In this study, we applied a comprehensive approach using multiple isolates and different culture conditions to gain insights into the cytotoxin

Table 1. *S. aureus* strains used in the study.

	MLST-SCCmec-spa-type or clonal complex	MRSA/ MSSA	lukSF-PV	lukED	hlgABC	lukGH	hla	Source of strain
Prototype wild-type strains								
TCH1516	ST8-IV-t622	MRSA	+	+	+	+	+	ATCC BAA-1717 TM
USA300 CA-MRSA								
NRS382	ST5-II-t002	MRSA	—	+	+	+	+	NARSA ^a
USA100 HA-MRSA								
MRSA252	ST36-II-t018	MRSA	—	+	+	+	— ^{**}	ATCC BAA-1720 TM
USA200 European HA-MRSA								
NRS385	ST8-IV-t064	MRSA	—	+	+	+	+	NARSA ^a
USA500 HA-MRSA								
NRS386	ST72-IVa-t126	MRSA	+	+	+	+	+	NARSA ^a
USA700 CA-MRSA								
MW2	ST1-IV-t125	MRSA	+	+	+	+	+	ATCC BAA-1707 TM
USA400								
Newman	ST254-t008	MSSA	—	+	+	+	+	Prof. Claire Poyart, Hôpital Cochin, Paris, France
H19	CC10	MRSA	—	+	+	+	+	Prof. Ashley Robinson, University of Mississippi Medical Center, USA
Live-stock MRSA								
Gene deletion mutant strains^b								
TCH1516 Δ hla/hlgABC/lukSF/lukED/lukGH or Δ all toxin mutant	ST8-IV-t622	MRSA	—	—	—	—	—	In house ^b
TCH1516 Δ lukGH	ST8-IV-t622	MRSA	+	+	+	—	+	In house ^b
TCH1516 Δ hla/hlgABC/lukSF/lukED or lukGH only	ST8-IV-t622	MRSA	—	—	—	+	—	In house ^b
TCH1516 Δ hla	ST8-IV-t622	MRSA	+	+	+	+	—	In house ^b
TCH1516 Δ hlgABC/lukSF/lukED/lukGH or hla only	ST8-IV-t622	MRSA	—	—	—	—	+	In house ^b
Human clinical isolates^c								
LA#3	ST1970-t065	MSSA	—	—	+	+	+	Lahey Medical Center ^c
LA#5	ST8-IV-t008	MRSA	—	+	+	+	+	Lahey Medical Center ^c
LA#16	ST81-t127	MSSA	—	+	+	+	+	Lahey Medical Center ^c
LA#45	ST121-t645	MSSA	—	+	+	+	+	Lahey Medical Center ^c
LA#48	ST45-t040	MSSA	—	—	+	+	+	Lahey Medical Center ^c
LA#66	ST5-II-t002	MRSA	—	+	+	+	+	Lahey Medical Center ^c
LA#132	ST72-t148	MSSA	—	+	+	+	+	Lahey Medical Center ^c
LA#164	ST188-t189	MSSA	—	+	+	+	+	Lahey Medical Center ^c
LA#178	ST8-t334	MSSA	—	+	+	+	+	Lahey Medical Center ^c
LA#186	ST5-II-t002	MRSA	—	+	+	+	+	Lahey Medical Center ^c
LA#217	ST8-IV-t008	MRSA	+	+	+	+	+	Lahey Medical Center ^c

Notes.

^{**}mutated (early STOP codon).^a Isolate was obtained through the Network on Antimicrobial Resistance in Staphylococcus Aureus (NARSA) program supported under NIAID/NIH contract HHSN272200700055C.^b *S. aureus* isogenic mutant strain generated in house based on the TCH1516 BAA-1717TM background.^c *S. aureus* isolates collected from ETA of ventilated patients. Stulik et al. AJRCCM, 2014.²⁸

expression profiles of *S. aureus* and to evaluate the protective effects of the two cytotoxin neutralizing mAbs, ASN-1 and ASN-2, on human cells.

Methods

Bacterial strains, culture conditions and preparation of culture supernatants

The USA300 CA-MRSA strain TCH1516 was obtained from ATCC (ATCC[®] BAA-1717TM). Isogenic mutants

lacking the *hla*, *hlgABC*, *lukED*, *lukSF-PV* and *lukGH* genes were generated in the TCH1516 background by homologous recombination as described previously.³⁵ Wild-type and mutant strains showed comparable growth characteristics based on OD_{600 nm} measurements and CFU counting on sheep blood agar plates.³⁸ 18 additional *S. aureus* strains used in this study are listed in Table 1.

For bacterial culture supernatants (CS) generated in CCY (3% wt/vol yeast extract, 2% Bacto-Casamino acids, 2.3% sodium pyruvate, 0.63% Na₂HPO₄, and 0.041% KH₂PO₄), BHI (Fluka), TSB (Corning) and RPMI-CAS

(RPMI-1640 Medium, Gibco; 1% Casamino acids, Amresco), overnight cultures obtained from a single colony were diluted to $OD_{600\text{ nm}}: 0.03$ in fresh culture medium and grown to stationary phase for 8 hours. All tested *S. aureus* strains reached similar bacterial densities within a given medium. CS were harvested by culture centrifugation at $5000 \times g$ at 4°C , followed by filter sterilization using $0.1 \mu\text{m}$ pore size PVDF syringe filters (Millipore).

Monoclonal antibodies

ASN-1 and ASN-2 were produced at Boehringer Ingelheim in stably transformed CHO cell lines. Monospecific antibodies against Hla, HlgB, and LukD were derived from the yeast expressed human IgG1 libraries selected with the respective recombinant monomers, as reported previously.³⁵ Human isotype control mAb (Motavizumab) and monospecific mAbs used for immunoblotting were produced in CHO-3E7 cells transiently transfected with mammalian expression plasmid pTT5 (National Research Council, Canada) encoding human antibody (IgG1) heavy and light chains using PEI MAXTM transfection reagent (Polysciences). IgGs were purified by Protein A or Protein G affinity chromatography and purities and monomeric states determined by SDS-PAGE and SEC, respectively, were both above 95%.

Recombinant toxins

S. aureus leukocidins were expressed based on the USA300 CA-MRSA genome sequences (TCH1516 strain), for LukGH also additional variants derived from MRSA252, H19 and MSHR1132 were generated, as described previously.^{38,39} Hla was expressed without tag and purified by cation exchange and size exclusion chromatography. Purity was controlled by SDS-PAGE.

Purification of peripheral white blood cells (WBCs) and PMNs

Human peripheral WBCs were isolated from heparinized blood using HetaSep (StemCell Technologies) gravity sedimentation, platelet removal by centrifugation and lysis of remaining erythrocytes using hypotonic sodium chloride solution (0.2%).

PMNs were isolated using Percoll Plus (GE Healthcare) gradient centrifugation, as described previously.³⁷ PMN purification resulted in cell purity of >95% determined microscopically by Giemsa staining as well as by flow cytometric analysis of forward- versus side-scatter properties. Cell viability was >98% for PMNs and WBCs

based on Trypan blue (Thermo Fisher Scientific) exclusion.

In vitro cytotoxicity and toxin neutralization assays

PMN cytotoxicity induced by recombinant toxins and culture supernatants was measured in 96-well format as described previously.^{35,38} PMNs (2.5×10^4 cells/well) were exposed to equimolar mixtures of S- and F-components for the bi-component toxins LukSF-PV, HlgAB, HlgCB, LukED or the co-expressed LukGH dimer in neutrophil medium (RPMI-1640 with 10% FCS (Sigma-Aldrich), 2 mM L-glutamine (Thermo Fisher Scientific)), for 4 hours at 37°C , 5% CO_2 . Cell viability was determined using the Cell Titer-Glo[®] Luminescent Cell Viability Assay Kit (Promega) according to manufacturer's instructions with a SynergyTM HT Multi-Mode Microplate Reader (BioTek). Percent cell viability after toxin exposure was calculated relative to mock treated cells. Data were analyzed by non-linear regression using Prism 6 (Graph Pad). The toxin monomers alone did not affect PMN viability in the concentration range tested. For toxin and CS neutralization assays, mAbs and toxins/CS were pre-incubated for 30 min at room temperature (RT) prior to addition to the target cells. A human IgG1 isotype control mAb was included in all experiments.

PMN infection assays with live bacteria

Overnight cultures of *S. aureus* grown in RPMI-CAS were diluted 1:50 and grown to mid-log phase ($OD_{600\text{ nm}}: 0.5$) at 37°C . Bacteria were harvested, washed with PBS to remove secreted toxins, re-suspended in RPMI-1640 with 10% FCS, 2 mM L-glutamine and 10 mM HEPES (Sigma) and added to 2.5×10^4 PMNs/well at different MOIs. Reactions were incubated for 90 min at 37°C and 5% CO_2 , followed by addition of Calcein-AM fluorescent viability dye (eBioscience) at a final concentration of $4 \mu\text{M}$ for an additional 30 min. Fluorescence ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 528 \text{ nm}$) was quantified on a SynergyTM HT Multi-Mode Microplate Reader (BioTek). Percent viability was calculated relative to mock-treated cells (100% viability).

For May-Grünwald-Giemsa staining PMNs at a concentration of $7.5 \times 10^6/\text{mL}$ were infected at MOI 50 in 4 mL round bottom tubes in 50 mg/mL human albumin (Biotest) in RPMI + 2 mM L-glutamine + 2 mg/mL sodium bicarbonate, pH ~ 7.5 (Sigma) and incubated for 2 hours at 37°C , 5% CO_2 . The cell suspension was then concentrated 10-fold by centrifugation at $280 \times g$ and re-suspended in fresh medium. $10 \mu\text{L}$ of each sample were placed onto glass slides, spread and allowed to air-dry. Cell smears were stained with May-Grünwald Solution (Sigma)

for 3 min and for 30 min with 10% Giemsa solution (Sigma), washed with dH₂O and air-dried. Images were taken with a ZEISS 100x Plan-APOCHROMAT (1.4 NA) on a ZEISS Imager M1 microscope equipped with Axio-CamMRc5 color camera.

Flow cytometry based surface staining of peripheral white blood cells

ASN100 mediated protection of human peripheral white blood cells was measured by flow cytometry in 96-well plate format. WBCs (1×10^6 /mL) were exposed to a pool of early stationary phase BHI cultures of TCH1516 and the six clinical isolates (indicated in Fig. 2B) in presence and absence of control antibody or toxin neutralizing mAbs. Following a 2 hour incubation at 37 °C and 5% CO₂, cells were pelleted by centrifugation (5 min, 1000 x g, RT) and stained with fluorochrome-labelled antibodies (BioLegend if not stated otherwise) specific for CD3 (clone HIT3a, APC), CD14 (clone 63D3, PE/Cy7), CD19 (clone HIB19, Alexa Fluor488) and CD56 (eBioscience; clone TULY56, eFluor 450) and the corresponding isotype control antibodies in Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific) supplemented with 0.5% BSA (Biomol) and 0.01% NaN₃ for 15 min at RT with constant mild agitation. Samples were measured with a CytoFLEX flow cytometer (Beckman Coulter) and data were analyzed with FCS Express version 5.0 (De Novo Software). Side-scatter and CD14 expression were used to discriminate lymphocyte, granulocyte and monocyte populations. Within the lymphocyte gate, T-cells, NK-cells, and B-cells were quantified based on expression of CD3, CD56, and CD19 respectively.

Human 3D lung tissue infection model

EpiAirwayTM tissues were obtained from MatTek. Upon delivery, the tissues were processed according to the supplier's protocol. For infection, PBS washed bacteria obtained from RPMI-CAS mid-log cultures were applied to the apical surface (MOI 100) in presence of either ASN-1, ASN-2, the mixture of these mAbs or an isotype control mAb (2 µM each) in 30 µL volumes. MABs were also added to the basal feeding medium at the same concentration. Following incubation for 24 hours at 37 °C and 5% CO₂, the basolateral medium was harvested for measuring LDH release using the CytoTox-ONETM Homogeneous Membrane Integrity LDH release Assay (Promega) and for subsequent measurement of PMN toxicity in a Cell Titer-Glo[®] Luminescent Cell Viability Assay Kit (Promega) as described above. Saponin in dH₂O (1%) was used as lysis control in LDH release assays. Bacteria on the apical surface of the tissue were

collected by three washing steps with 300 µL PBS and serial dilutions were plated on sheep blood agar plates for CFU determination. The washed tissues were fixed overnight with a total of 2 mL 4% paraformaldehyde (Sigma) at 4 °C, embedded in paraffin and 1 µm sections were stained with hematoxylin and eosin at the automated slide stainer Gemini AS (Histocom). Images were taken with a ZEISS EC Plan-Neofluar 20x/0.50 objective on a ZEISS Imager M1 microscope equipped with Axio-CamMRc5 color camera.

Immunoblotting to detect cytotoxin expression

Early stationary phase CS generated in different media were separated by SDS-PAGE under reducing conditions. Proteins were then transferred to PVDF (Hla, LukG and LukS-PV) and nitrocellulose membranes (LukD, HlgB) using a Trans-Blot Turbo device (BioRad). Hla, LukD and HlgB expression was assessed using monospecific human antibodies, LukS-PV was detected with a mouse monoclonal antibody (IBT Bioservices) and LukG expression was measured using a rabbit anti-LukB polyclonal Ab (IBT Bioservices). Specific reactivity to the target antigens and lack of cross-reactivity with other toxins was proven by immunoblotting using recombinant Hla, LukS-PV, LukF-PV, HlgA, HlgB, HlgC, LukE, LukD and LukGH. Antibody binding was detected using anti-human, rabbit or mouse HRP-conjugated goat F(ab')₂ fragments (Southern Biotech) with the enhanced chemiluminescence (ECL) immunoblotting detection reagent (GE Healthcare) at an ImageQuant LAS 4000 (GE Healthcare) Imaging station.

Live-dead cell staining

PMNs (1×10^5 cells/well) were exposed to *S. aureus* CS and mAbs in presence of 4 µM Calcein AM and 2 µM EthD-1 (LIVE/DEAD[®] Viability/Cytotoxicity Kit, Life Technologies) in 8-well µ-slides (Ibidi) in neutrophil medium. PMN survival was monitored by time-lapse microscopy using a Cell-R Live Imaging System (Olympus). Pictures were taken in 1 minute intervals for a total of 90 min and exported as movies with a 3 frames per second rate.

Results

Inactivation of the five leukocidins of *S. aureus* by two toxin neutralizing human mAbs, ASN-1 and ASN-2

We measured leukocidin-mediated cytotoxicity towards polymorphonuclear cells (PMNs) in cell based viability

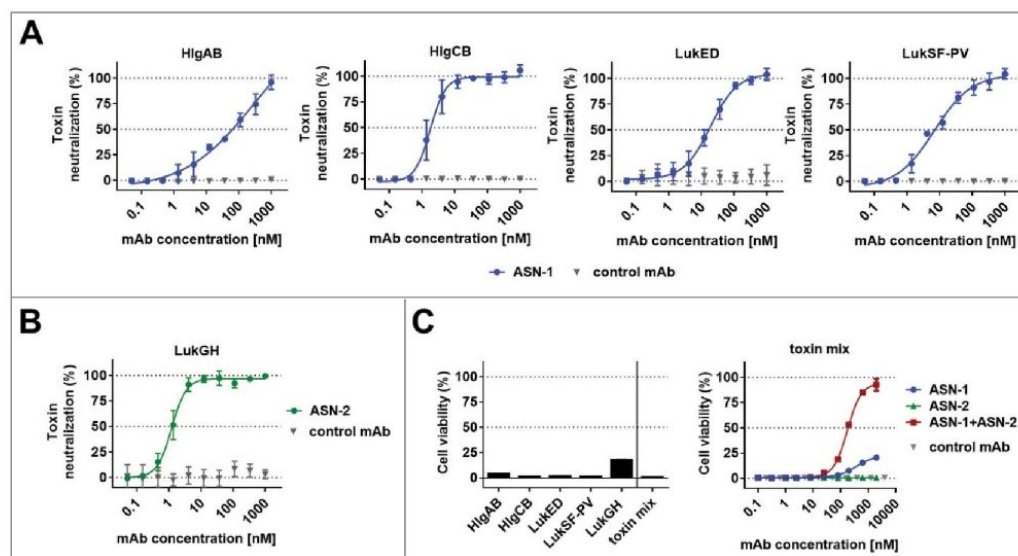


Figure 1. Neutralization of recombinant leukocidins by ASN-1 and ASN-2. A: Neutralization of recombinant HlgAB (10 nM), HlgCB (7.5 nM), LukED (10 nM) and LukSF-PV (5 nM) by ASN-1. B: Neutralization of LukGH (1 nM, TCH1516 LukGH) by ASN-2. C: left panel: Effect of individual toxins as in A, except for LukGH (1.875 nM each of the four LukGH variants expressed based on the MRSA252, MSHR1132, H19 and TCH1516 sequences) and a mixture thereof on PMN viability; right panel: Neutralization of a mixture of recombinant toxins by ASN-1, ASN-2 or ASN100 (1:1 mixture of ASN-1 and ASN-2). Antibody concentrations shown refer to individual mAbs. PMN viability was determined by luminescent quantification of cellular ATP levels. Data are derived from at least two independent experiments and shown as mean $\pm$ SEM.

assays by luminescent detection of ATP. ASN-1 preserved the viability of human PMNs exposed to recombinant HlgAB, HlgCB, LukED, and LukSF-PV (Fig. 1A), and ASN-2 fully neutralized recombinant LukGH (Fig. 1B).

Since *S. aureus* typically expresses several leukocidins, up to all five in some strains, antibody potency was also assessed in the presence of all five leukocidins. A mixture of recombinant toxins was used in cytotoxin neutralization assays at concentrations that reduced PMN viability by at least 80% (Fig. 1C). In these experiments, neither ASN-1 nor ASN-2 was able to inhibit toxicity alone; however, the equimolar combination of the two mAbs, called ASN100, was highly effective in blocking all five leukocidins and fully preserved cell viability.

S. aureus isolates differ in their overall PMN toxicity and leukocidin expression profiles

To characterize the effect of naturally-produced cytotoxins on neutrophils and to measure the effectiveness of the two toxin neutralizing antibodies, we exposed PMNs to *S. aureus* culture supernatants containing the secreted toxins. We used seven *S. aureus* isolates representing different genotypes: Four MRSA strains including a genome sequenced prototype USA300 CA-MRSA strain (TCH1516) and three MSSA strains with different

sequence- (ST) and *spa*-types (Table 1). Five of the seven strains were collected from mechanically ventilated patients.²⁸ To assess the contribution of the leukocidins to PMN toxicity in this assay, we generated a gene deletion mutant of the TCH1516 strain that lacked all five leukocidin genes (and additionally *hla*). Bacteria were grown to early stationary phase in four different growth media: three rich bacterial culture media, CCY, BHI, and TSB, most often used for culturing *S. aureus*, and RPMI, a mammalian cell culture medium (supplemented with casamino acids), to mimic *in vivo* conditions, such as low iron and nutrient content. The growth kinetics and optical densities after 8 hours growth when CSs were collected were comparable (Fig. S1). PMN toxicity was determined by serial dilution of the culture supernatants (CS) and expressed as the CS dilution that still resulted in a 50% reduction in cell viability (EC_{50}) (example shown in Fig. 2A). Importantly, the CSs of the mutant strain lacking all six pore forming toxins did not display measurable EC_{50} , and induced maximum 25% loss of viability at the lowest CS dilution. We also determined that Hla did not have an impact on viability of human PMNs in this assay (tested with recombinant Hla and CS of the TCH1516 Δ hlgABC/lukED/lukSF-PV/LukGH mutant strain; data not shown). PMN toxicity in this *in vitro* model is therefore mainly driven by the leukocidins. Great differences were observed with the seven *S. aureus*

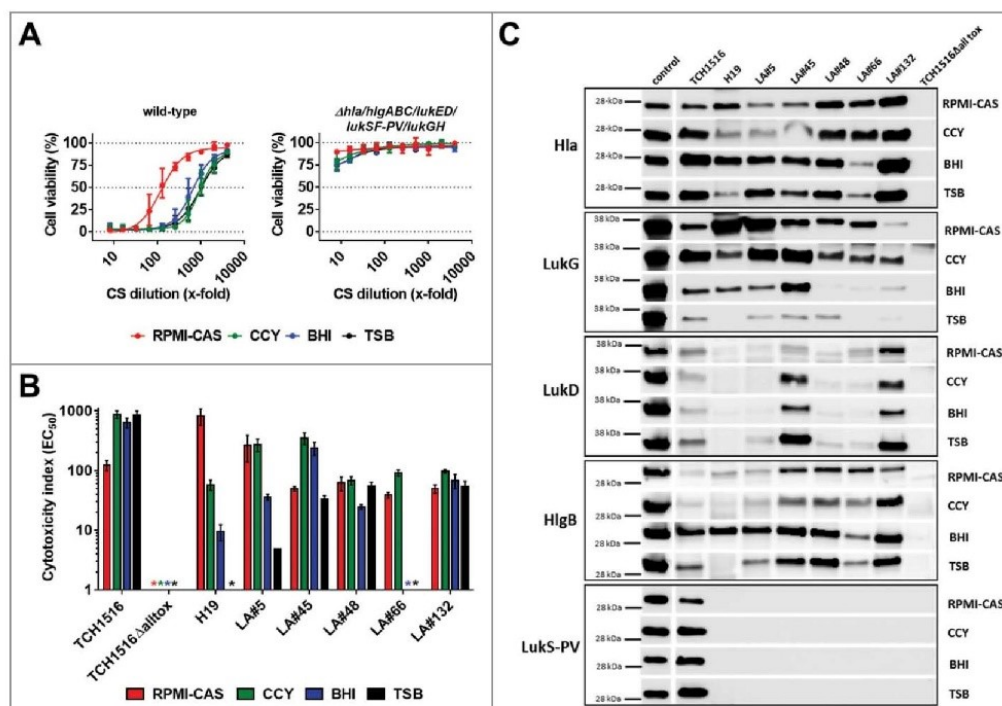


Figure 2. Toxin expression profiles and neutrophil toxicity of *S. aureus* isolates. **A:** Viability of human PMNs after exposure to serially diluted culture supernatants (CS) of USA300 CA-MRSA TCH1516 strain (left panel) and its isogenic $\Delta hla/hlgABC/lukED/lukSF-PV/lukGH$ mutant ("TCH1516 Δ alltox", right panel) grown in the indicated growth media. **B:** PMN toxicity of seven *S. aureus* strains expressed as CS dilution at the EC₅₀. Toxicity too low for EC₅₀ calculation. PMN viability was determined by luminescent quantification of cellular ATP levels. Data are expressed as mean $\pm$ SEM of three independent experiments. **C:** Immunoblot analysis of CS of seven *S. aureus* strains grown in the indicated media; control, recombinant toxin (0.1 μ g/lane).

strains in the same growth medium or with the same strain in different broths, with EC₅₀ values ranging from 5 to approximately 900 (Fig. 2B). For example, while the LA#48 and LA#132 clinical isolates showed comparable toxicity when grown in the four different media, leukocidin expression of the livestock-associated MRSA strain H19 and the LA#66 isolate was greatly affected (>100-fold difference). Notably, CS generated with these two strains in TSB were not toxic to PMNs. All growth media considered, the *lukSF-PV* USA300 CA-MRSA strain TCH1516 displayed the highest PMN toxicity.

Immunoblot analysis with cytotoxin specific antibodies revealed greatly variable expression levels of the individual leukocidins by the seven different *S. aureus* strains in the four different growth media tested (Fig. 2C), supporting the results of the PMN toxicity assays. LukG expression was detectable with all strains, with highest levels in RPMI-CAS and CCY, and low levels in TSB. HlgB levels were highest and comparable among strains in BHI medium, while three strains displayed lower levels in the other three media. Although *lukED* was present in six of the seven characterized isolates, only TCH1516, LA#45 and LA#132 showed detectable LukD expression

in all four media. LukSF-PV expression was expected only with the TCH1516 strain based on the presence of *lukSF-PV* (Table 1). This toxin was detected in all four growth media, with slightly lower intensity in RPMI-CAS, compared to CCY, BHI and TSB. Since ASN-1 also binds Hla, which can compete for binding to the leukocidins, we also determined Hla levels in the bacterial CSs. Compared to the leukocidins, Hla levels were more uniform across the different culture supernatants, but still influenced by the growth media in a strain-dependent manner (Fig. 2C).

Prevention of neutrophil lysis by *S. aureus* culture supernatants requires both ASN-1 and ASN-2

We measured neutralization of *S. aureus* CS in the presence of ASN-1, ASN-2 or both mAbs at 1 μ M (150 μ g/mL) concentration in several *in vitro* assays.

First, we utilized the ATP-based luminescent cell viability assay with human PMNs to assess the contribution of the individual mAbs to leukocidin neutralization. The TCH1516 strain grown in RPMI-CAS demonstrated almost 100% PMN cytotoxicity (using 8-fold diluted CS)

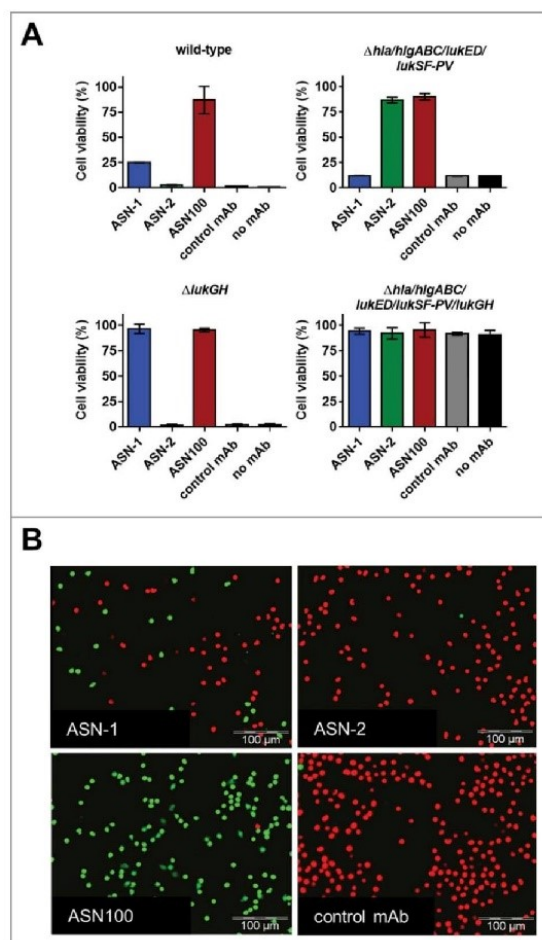


Figure 3. Synergistic effect of ASN-1 and ASN-2 in neutralizing native *S. aureus* cytotoxins in bacterial culture supernatants (CS). A: Human PMNs were exposed to CS of TCH1516 (at 8x dilution) and isogenic toxin gene deletion mutants grown in RPMI-CAS, in the presence of control antibody, ASN-1, ASN-2 or ASN100 (1 μ M each). Data are shown as mean $\pm$ SEM of two independent experiments. Viability was determined by luminescent quantification of cellular ATP levels. B: Calcein-AM/EthD-1 live/dead cell staining of human neutrophils after exposure to CS of TCH1516 (at 10x dilution) in the presence of ASN-1, ASN-2 and ASN100 (1 μ M each) for 90 min. Live cells stained green, dead cells appear in red.

in presence of an isotype control mAb (Fig. 3A, upper left panel). ASN-1 could partially inhibit toxicity while ASN-2 alone was ineffective. The equimolar mixture of the two mAbs, ASN100, preserved PMN viability (Fig. 3A, upper left panel). PMNs exposed to CS of the TCH1516 $\Delta lukGH$ mutant were fully protected by ASN-1 (Fig. 3A, lower left panel), while those exposed to the CS of the TCH1516 $\Delta hla/hlgABC/lukED/lukSF-PV$ strain remained viable in the presence of ASN-2 (Fig. 3A, upper right panel). The CS of the TCH1516

strain lacking all toxin genes (TCH1516 $\Delta hla/hlgABC/lukED/lukSF-PV/lukGH$) did not affect PMN viability (Fig. 3A, lower right panel).

Detection of pore formation by microscopy using Calcein-AM and EthD-1 for live/dead cell staining, mirrored the results obtained with the ATP-based luminescent assay (Fig. 3B). Time-lapse microscopy revealed that cell death in the control and ASN-2 containing samples occurred within minutes after exposure to TCH1516 CS. This effect was partially inhibited by ASN-1 and fully prevented in presence of both mAbs (Fig. 3B, Supplementary Movie File S1).

PMNs exposed to 28 *S. aureus* CS (seven strains grown in the four different media) were all protected by the equimolar mixture of ASN-1 and ASN-2 (ASN100), although greatly different patterns of protection were observed with the individual antibodies (Fig. 4A, Supplementary Fig. S2). Toxicity of the LukSF-PV-expressing USA300 strain was partially neutralized by ASN-1, but not at all by ASN-2, independent of the growth medium (Fig. 4A, upper panel). The CS of LA#5 (expressing high amounts of LukGH in RPMI, CCY and BHI medium) was neutralized with high potency by ASN-2 alone (Fig. 4A, middle panel). Neutralization of the LA#45 isolate displayed a mixed pattern dependent on the growth media (Fig. 4A, lower panel), which correlated well with the immunoblotting data (Fig. 2C). The high LukG production by this strain in RPMI medium (relative to LukD and HlgB) resulted in ASN-2 dominance, but a strong need for ASN-1 was observed in TSB medium (high LukD and HlgB levels, low LukGH). The BHI and CCY CSs contained comparable amounts of these three toxins, therefore, both mAbs were required for protection of PMNs (Fig. 4A, lower panel).

RPMI-CAS supernatants of a larger panel of *S. aureus* isolates, including the major MRSA pulsotypes USA100, USA200 (MRSA252), USA400 (MW2), USA500, and USA700, the Newman strain and 6 clinical isolates were also characterized (Table 1, Fig. 4B). Neutralization of PMN toxicity of *lukSF-PV*⁺ strains (TCH1516, LA#217, NRS386 and MW2) was generally dependent on both antibodies, while toxin activity mediated by *lukSF-PV*⁻ strains could be partially or fully inhibited by ASN-2 alone. Notably, we did not encounter a single *S. aureus* strain during these studies for which complete neutralization of toxicity was independent of ASN-2 in the low iron containing culture medium.

We also modeled the effect of different ASN-1 and ASN-2 ratios on PMN protection using CSs of various strains grown in different media. As the amount of any of the two mAbs was reduced in the neutralization assays (while one was kept constant), declining protection levels were observed with most supernatants (examples with

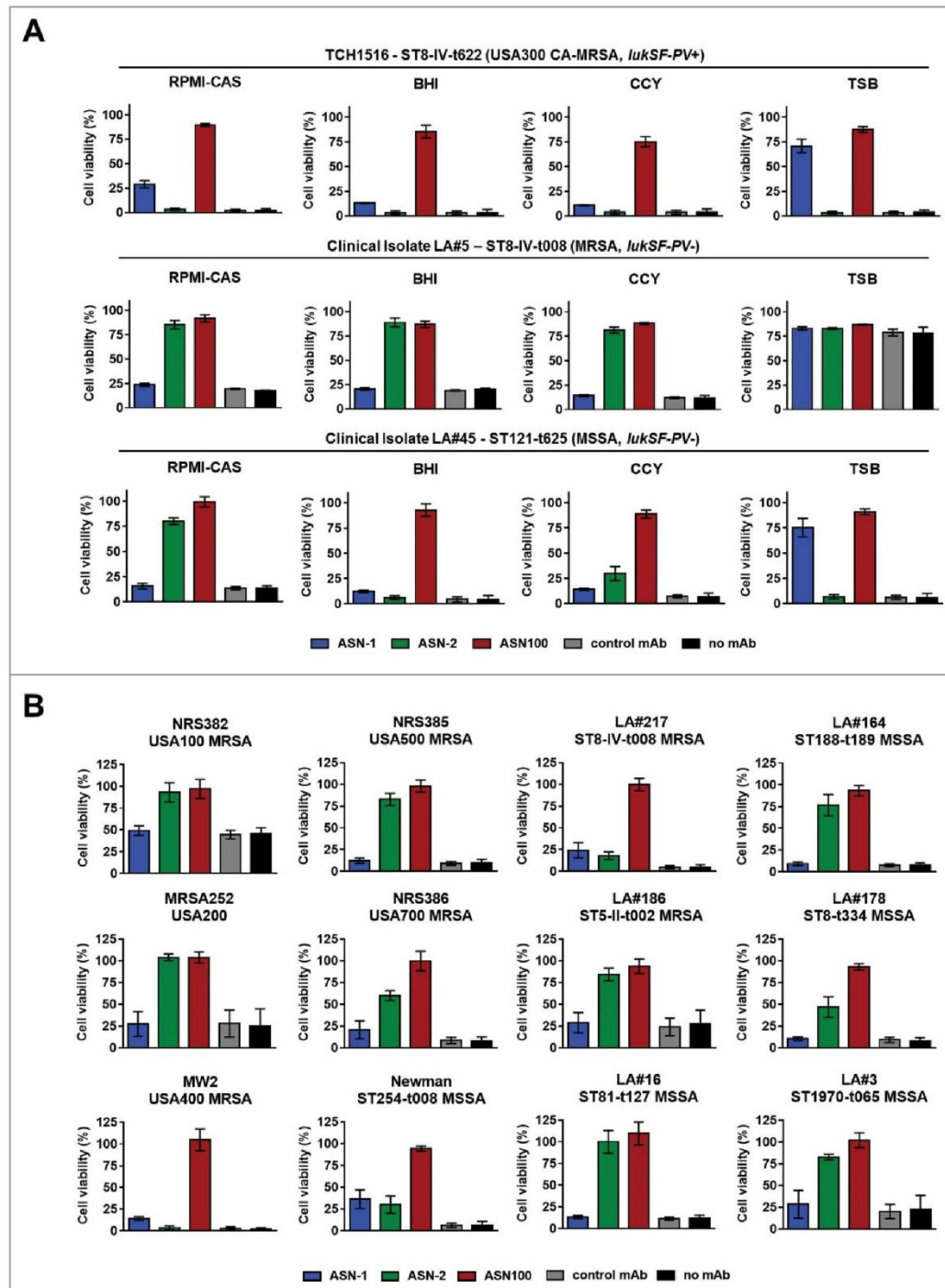


Figure 4. Individual contributions of ASN-1 and ASN-2 to PMN protection are strain and growth condition dependent. *S. aureus* isolates were grown in the indicated media, culture supernatants (at 16x dilution) were tested for PMN toxicity in the presence of ASN-1, ASN-2 and ASN100. A: Three representative isolates grown in different media. B: Six prototype strains and six clinical isolates grown in RPMI-CAS. PMN viability was determined by luminescent quantification of cellular ATP levels. MAbs were used at 1 μ M each. Data are shown as mean $\pm$ SEM of three independent experiments.

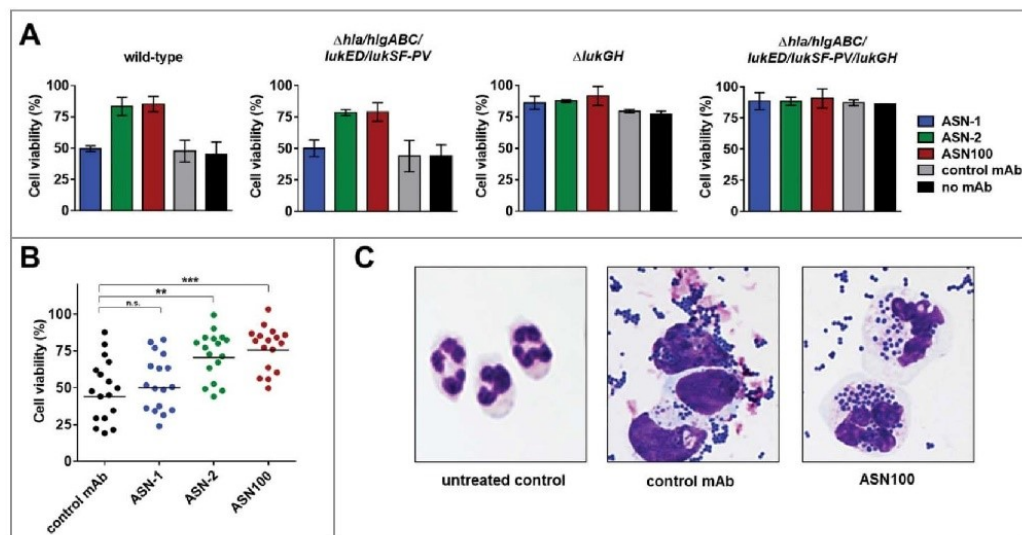


Figure 5. Effect of ASN100 during PMN *ex vivo* infection. Human PMNs were infected with different *S. aureus* strains at a MOI 50 for 2 hours. PMN viability was determined using a Calcein-AM viability dye. **A:** TCH1516 and isogenic mutant strains, mAbs used at 1 μ M each. **B:** Data summary of TCH1516 and 16 additional clinical isolates, ** $p \leq 0.01$ and *** $p \leq 0.001$ using one-way ANOVA with Dunn's multiple comparison test. Data are presented as mean $\pm$ SEM of two independent experiments. **C:** May-Grünwald Giemsa staining of human PMNs 2 hours after infection with TCH1516, 100x magnification.

TCH1516 and LA#132 strains grown in RPMI-CAS and CCY are shown in Fig. S3).

Since the presence of phagocytic cells upregulates leukocidin expression by *S. aureus*⁴⁰ and PMN activating signals modulate cytotoxin susceptibility,³⁸ we also assessed the relative contribution of ASN-1 and ASN-2 to preserving neutrophil viability in *ex vivo* infection experiments. PMNs were infected with different *S. aureus* strains at a starting multiplicity of infection (MOI) of approximately 50. PMN viability was determined after 2 hours with the fluorescent viability dye, Calcein-AM. The greatly reduced ability of the TCH1516 Δ lukGH strain to kill PMNs, and the retained toxicity of the TCH1516 Δ hla/hlgABC/lukED/lukSF-PV strain that was comparable to that induced by the wild-type strain, indicated a largely LukGH-dependent cytotoxic effect (Fig. 5A). In concordance with this, ASN-1 contributed little to the protection of PMNs from TCH1516, while ASN-2 alone was very efficient in maintaining viability comparable to ASN100. 16 other *S. aureus* strains (also tested in the culture supernatant mediated toxicity assay) were evaluated and found to induce 15% to 80% PMN cytotoxicity (Fig. 5B). In most cases, ASN-2 alone displayed protection levels comparable to ASN100, while ASN-1 alone was only marginally beneficial. These data suggest that under these experimental conditions, PMN death was largely mediated by LukGH with all *S. aureus* isolates tested.

Microscopic analysis of May-Grünwald-Giemsa stained PMNs exposed to TCH1516 live bacteria for two

hours revealed that control samples (treated with isotype control mAb) showed swollen nuclei, altered cytoplasmic staining and few, if any, intracellular *S. aureus*, while in the presence of ASN100 cell morphology was preserved and neutrophils were able to engulf bacteria in large numbers (Fig. 5C). Notably, a similar outcome was achieved with the gene deletion mutant lacking the genes for all leukocidins and Hla in absence of neutralizing antibodies (Fig. S4).

ASN100 prevents lysis of all human white blood cells susceptible to *S. aureus* leukocidins

Although neutrophils are considered to be the major target cells of *S. aureus* leukocidins (all five are highly active on this cell type), lysis of other leukocyte types such as monocytes, lymphocytes and NK cells is also expected based on leukocidin receptor expression profiles.¹¹ To assess the protective effect of ASN100 for these cell populations, we isolated peripheral white blood cells from fresh human blood and exposed them to CS pools of the seven strains characterized in Fig. 2. The different cell types were phenotyped by flow cytometry using side scatter properties in combination with cell surface markers (Fig. 6 and Fig. S5). Exposure to *S. aureus* CS almost completely eliminated granulocytes, monocytes, and NK-cells populations, and significantly reduced the number of T-lymphocytes and B-lymphocyte counts (Fig. 6). Similarly to the results obtained with purified PMNs in the viability assays, com-

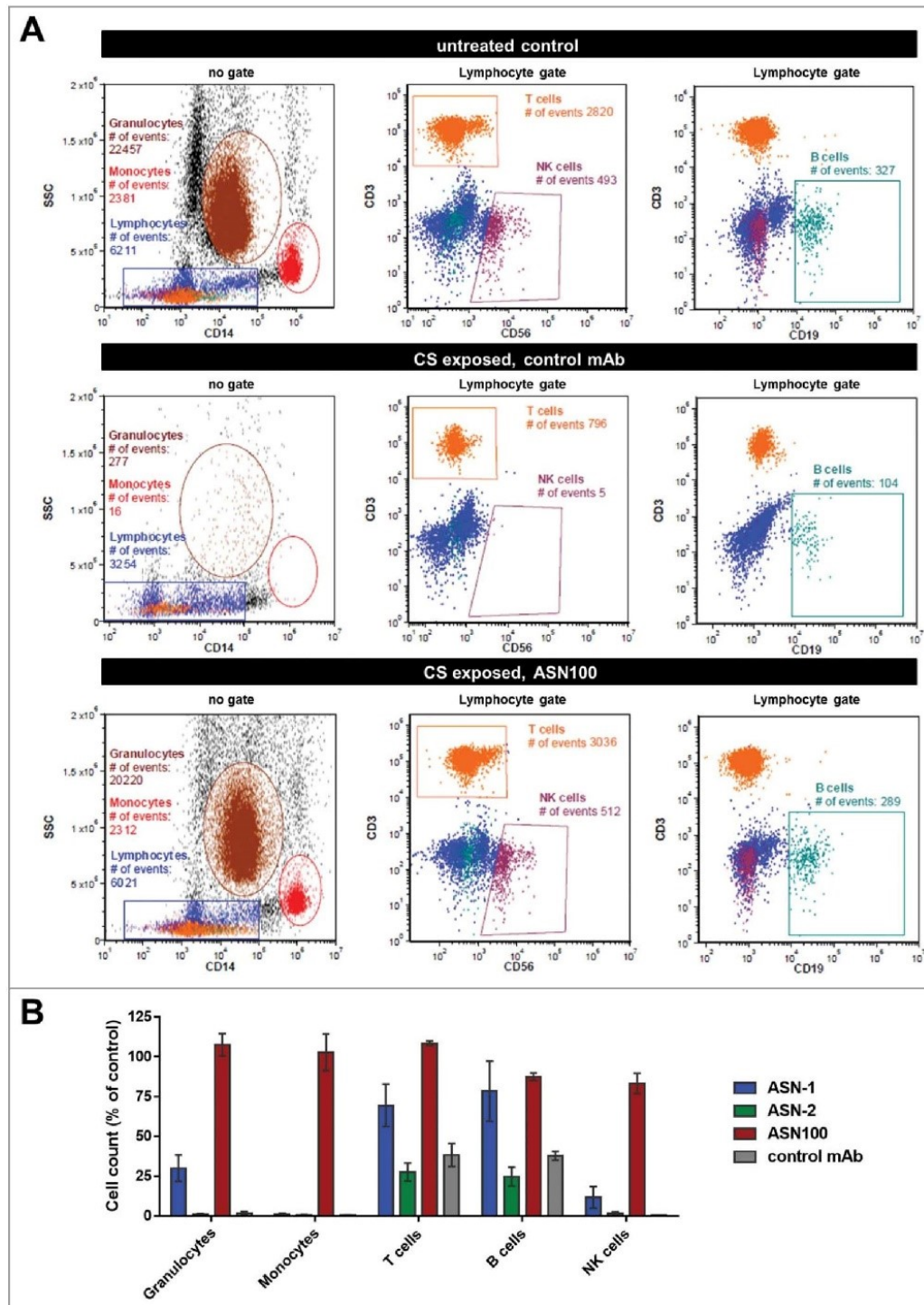


Figure 6. Protection of white blood cells by ASN100. Human peripheral WBCs were exposed to pooled BHI CS fractions of seven *S. aureus* strains for 2 hours and then stained with fluorescent antibodies specific for CD3, CD56, CD14, and CD19. Side-scatter and CD14 expression were used to discriminate lymphocyte, granulocyte and monocyte populations. T-cells, NK-cells and B-cells in the lymphocyte gate were further subtyped based on CD3, CD19 and CD56 expression. **A:** Representative plots showing untreated control cells and CS-exposed cells in presence of control mAb and ASN100. **B:** Data summary of 3 independent experiments using ASN-1, ASN-2 and ASN100. Data are shown as mean $\pm$ SEM.

plete protection of granulocytes, monocytes, T-cells and NK cells was dependent on the simultaneous presence of both, ASN-1 and ASN-2 (Fig. 6B). B-cell lysis was largely preventable with ASN-1 alone (Fig. 6B and Fig. S5). Importantly, ASN100 was effective in preserving the viability of all cell types analyzed.

Protective effects of ASN100 in an in vitro 3D airway epithelial tissue model infected with *S. aureus*

The cytoprotective effect of ASN-1 and ASN100 via Hla-neutralization was tested in a 3D- primary human respiratory epithelial tissues culture model (EpiAirway™, MatTek). These tissues consist of normal, human-derived tracheal/bronchial epithelial cells that are highly differentiated containing cilia and tight junctions and retain properties of normal respiratory tract epithelial tissue including active mucus secretion. Tissues were

infected with the *S. aureus* TCH1516 strain using an MOI of 100 on the apical side for 24 hours in the presence of ASN-1, ASN-2, ASN100, or an isotype-matched control mAb. Hematoxylin-eosin staining revealed complete tissue destruction by *S. aureus* in samples with the control antibody and ASN-2, while ASN-1 and ASN100 treated samples displayed fully preserved tissue integrity (Fig. 7A). LDH release, measured in the basal feeding medium mirrored the microscopy data confirming full lysis of cells by *S. aureus* (comparable to saponin-treated samples), except when cells were co-incubated with ASN-1 or ASN100 (Fig. 7B). The lysis of epithelial cells was dependent on Hla, as the *hla*-deletion mutant *S. aureus* strain was not toxic, while the leukocidin gene deletion mutant strain was indistinguishable from the wild-type strain (Fig. 7C). Interestingly, we observed great reduction in bacterial counts of ASN-1 and ASN100 protected samples relative to control or ASN-2

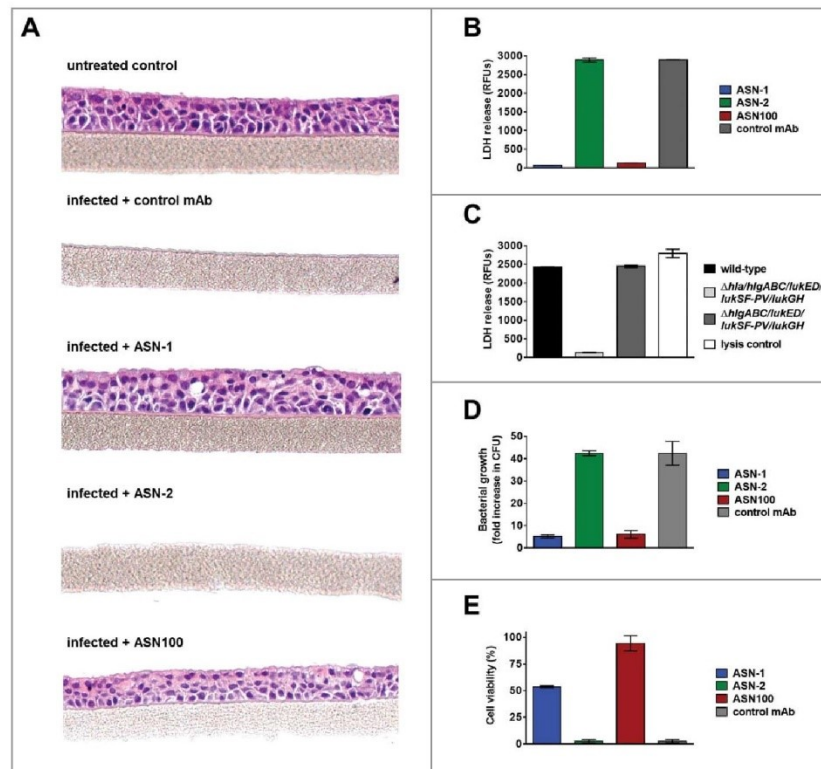


Figure 7. ASN100 prevents Hla-mediated tissue damage in a human 3D lung tissue infection model. EpiAirway™ tissues were infected with *S. aureus* TCH1516 at MOI 100 for 24 hours in the presence of control mAb, ASN-1, ASN-2 or ASN-100 (2 μ M each). **A:** H&E staining of tissues. **B:** LDH release measured from basolateral compartment. **C:** LDH release measured with wild-type and gene deletion mutant TCH1516 strains in absence of mAbs. **D:** Relative increase in bacterial CFUs determined in the apical compartment. **E:** Viability of human neutrophils exposed to toxins produced in the control mAb sample in the presence of ASN-1, ASN-2 and ASN100 (1 μ M each). Error bars indicate mean $\pm$ SEM from duplicate measurements.

treated samples (Fig. 7D). This is likely due to the growth enhancing effects of nutrients released from lysed cells, which is prevented in the presence of ASN-1.

Since no white blood cells are present in the MatTek EpiAirwayTM model, Hla neutralization was sufficient to block *S. aureus* mediated toxicity. To indirectly assess leukocidin toxicity and ASN100 mediated protection of neutrophils, we collected the basolateral fluid of the tissues infected with *S. aureus* in the absence of toxin-neutralizing antibodies and tested for toxicity towards human neutrophils in presence of ASN-1, ASN-2 or ASN100. As bacteria cannot pass through the seeding membrane, only soluble molecules (such as toxins) reach the basolateral compartment. Performing neutralization assays with the harvested media containing the *in situ* produced toxins from the infected tissues we observed a similar pattern of PMN toxicity and ASN100 mediated protection as seen in bacterial culture media, namely partial neutralization with ASN-1, and full protection by ASN100 (Fig. 7E). This confirmed that *S. aureus* produced both Hla and multiple leukocidins during infection of the lung tissues and that all toxins could be neutralized by ASN100.

Discussion

Given the high redundancy, species specificity, and variability of expression of *S. aureus* pore-forming cytotoxins, we applied a comprehensive approach to examine their effects on human target cells and to evaluate the potency of two human mAbs specific for alpha-hemolysin and five leukocidins in six different *in vitro* models. First, human PMNs, containing mainly neutrophils were exposed to the mixture of recombinant leukocidins. The two mAbs – ASN-1 that neutralizes four of the five leukocidins (in addition to Hla) and ASN-2 targeting the 5th leukocidin, LukGH – were both required to prevent PMN cytotoxicity. Next, we characterized the leukocidin expression profiles of seven *S. aureus* isolates with different genetic backgrounds grown in four different culture media commonly used for *in vitro* culturing of *S. aureus*. Immunoblotting revealed variable expression levels of the individual leukocidins of the different strains that were influenced by the culture media. RPMI medium (optimized for mammalian cell cultures) is considered to be more appropriate to imitate *in vivo*-like conditions than rich bacterial culture media. Its low iron and nutrient content and physiologic ionic strength are expected to reflect the situation in plasma and tissue fluids. This assumption is supported by high promoter activity of the leukocidin genes in such media.⁴¹ The variable leukocidin expression in response to the different culture media was reflected by the PMN toxicity of the culture

supernatants. Similar findings were made by quantitative mass-spectrometry and correlating individual leukocidin expression levels to the degree of PMN toxicity measured in RPMI versus rich culture medium.⁴²

Cytotoxin expression is subject to complex regulation and influenced by environmental conditions. As seen with most secreted virulence factors, the cytotoxins are expressed mainly in the stationary growth phase *in vitro*. The global regulator Agr that is part of the quorum sensing (bacterium density) mechanism, is the master regulator of expression, but many other regulators, such as Sae, Rot, Sar, Rsp, and metabolic sensors have also been shown to modulate the expression of *S. aureus* cytotoxins.^{43–46} Mutations and/or variations in transcriptional regulators have been described in clinical *S. aureus* isolates. For example, a unique *saeS* allele was reported that overrides cell-density dependent SaeR and LukSF-PV expression in ST30 CA-MRSA isolates, resulting in a higher level of LukSF-PV in early log phase.⁴⁷ Several studies report that *S. aureus* exposed to the host environment (human blood cells, infected animals in pneumonia model, surfactant, etc.) greatly up-regulates expression of *hla* and the leukocidin genes.^{40,41,48–50} Moreover, we found that exposure to inflammatory stimuli, such as LPS, *S. aureus* culture supernatant (peptidoglycan and delta-toxin), and IL-8, activates PMNs and alters their sensitivity towards the individual leukocidins, rendering LukGH the most dominant leukocidin *in vitro* due to up-regulation of its receptor, CD11b.³⁸ Even without PMN stimulation, LukGH has been reported to be prominent among leukocidins in the culture supernatants of *S. aureus*.^{42,51,52}

We found that ASN-1 or ASN-2 alone had variable effects on PMN viability upon exposure to the culture supernatants of 19 *S. aureus* strains, ranging from no effect to partial efficacy, and in some cases even full protection with ASN-2. Importantly, irrespective of the genetic background or culture medium used, the combination of the two mAbs, ASN100, was always sufficient to prevent PMN cytotoxicity. These data suggest that preventing immune cell damage necessitates the simultaneous neutralization of all leukocidins. *In vitro* infection of human PMNs with *S. aureus* revealed that LukGH was responsible for the majority of the killing effect and ASN-2 alone was highly protective. Microscopic analysis of PMNs protected by ASN100 detected intact cells that were able to engulf *S. aureus*; these intracellular bacteria were not seen in the controls. These data suggest that not only PMN viability but also phagocytic activity was maintained with ASN100.

Since leukocidins do not only target neutrophils, but also monocytes, macrophages and lymphocytes,^{9,11} we confirmed ASN100 efficacy and the requirement for both mAbs with these important cell populations.

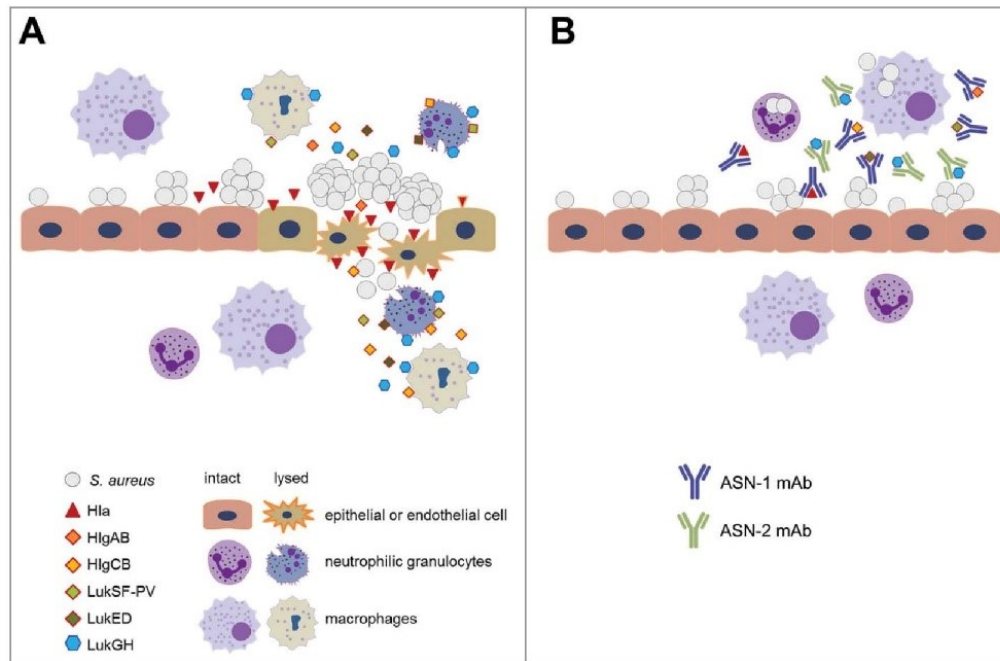


Figure 8. Proposed model for the role of *S. aureus* cytotoxins in pathogenesis and mode of action of ASN100. A: As bacterial density increases on mucosal surfaces, *S. aureus* secretes up to six cytolytic toxins. Alpha-hemolysin (Hla) lyses epithelial and endothelial cells allowing invasion to otherwise sterile body parts (e.g. lung, connective tissue) and promoting *S. aureus* disease (e.g. pneumonia, deep tissue infection). Up to five different leukocidins that primarily attack phagocytic cells, such as alveolar and tissue macrophages and granulocytes are also released, leading to weakened host defense and slower elimination of *S. aureus*. B: ASN-1 that neutralizes Hla and four leukocidins and ASN-2 that is specific for the fifth leukocidin (LukGH) inhibit cytotoxicity of *S. aureus*, which allows preservation of tissue integrity and immune defense during infection.

Hla-mediated pathogenesis was mimicked by infecting primary human mucociliary tracheal/bronchial epithelial tissue cultured at the air-liquid phase with *S. aureus*. We observed full protection of epithelial cell morphology and prevention of LDH release by ASN-1 due to its Hla-neutralizing activity. Moreover, reduced growth of *S. aureus* in the presence of ASN-1 was also evident, most likely due to limited access to nutrients from lysed epithelial cells.

Based on the *in vitro* data presented here and the high *in vivo* efficacy in rabbit models,^{15,54} we propose that ASN100 treatment greatly reduces *S. aureus* virulence by preventing Hla-mediated tissue damage and subsequently bacterial invasion, and by preserving phagocyte function, enabling the human host to efficiently control bacterial growth (Fig. 8). In addition, prevention of neutrophil killing is expected to reduce inflammation and edema, especially in the lung, where recruited and subsequently lysed phagocytes damage the lung by releasing the contents of cytotoxic granules and/or reactive oxygen species.¹⁷ As antibiotics do not address these important virulence determinants, and are even implicated in enhancing cytotoxin production by *S. aureus*,⁵⁵ adjunct therapy in addition to prophylaxis or preemptive applications is justified.

The limitation of our study is related to the lack of data on *in vivo* cytotoxin expression levels in patients. The increase in cytotoxin specific antibodies during convalescence from invasive *S. aureus* infections provide evidence that these virulence factors are indeed expressed in human *S. aureus* disease.^{56,57} Notably, the greatest increase in anti-cytotoxin antibody levels was seen with LukGH (LukAB).⁵⁷

Although ASN-1 alone is able to neutralize five different cytotoxins, while ASN-2 can inhibit only one, the function of ASN-2 is crucial for the protection of phagocytic cells due to the dominant role of LukGH, especially under iron-restricted conditions and upon exposure of PMNs to inflammatory stimuli.³⁸ Consistent with the importance of both mAbs and their synergistic mode of action, changing the ratios of ASN-1 or ASN-2 by decreasing one component and keeping the other constant was inferior in overall protective potency. Facing the highly variable leukocidin expression profiles across clinical isolates, our approach is to provide sufficiently high mAb levels in patients, not only in the plasma but also in epithelial lining fluids. In the ongoing Phase 2 clinical trial (NCT02940626), mechanically ventilated patients who become heavily colonized with *S. aureus*

(based on microbiological analysis of ETA samples) receive equal amounts of ASN-1 and ASN-2 (1800 mg each). Based on the pharmacokinetic analysis from a healthy volunteer study, this ASN100 dose is expected to result in approximately 450 $\mu\text{g/mL}$ initial and $>150 \mu\text{g/mL}$ plasma levels of ASN-1 and ASN-2 for at least 2 weeks following intravenous administration.⁵⁸

The recognition of the toxin-driven pathogenesis of *S. aureus* started almost 100 years ago and led to passive immunization trials in patients with complicated skin infections.^{59,60} Recent clinical studies with anti-*S. aureus* vaccines and passive immunization include or completely rely on anti-toxin approaches.^{5,61} These forthcoming studies will reveal whether inactivation of cytotoxins can result in prevention or amelioration of *S. aureus* infections in humans.

Disclosure of potential conflicts of interest

All authors, except SM, declare potential conflict of interest as they are employees of Arsanis Biosciences GmbH and shareholders in Arsanis, Inc.

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Manuscript I

**Assessing the function of pneumococcal neuraminidases NanA, NanB and NanC in
in vitro and *in vivo* lung infection models using monoclonal antibodies**

“If opportunity doesn’t knock, build a door.”

Milton Berle

Background and summary:

Streptococcus pneumoniae isolates express up to three neuraminidases (sialidases), NanA, NanB and NanC. In contrast to *S. aureus* leukocidins, these enzymes are not involved in killing of host cells. The major focus of research has hitherto been on NanA. It is important in bacterial sugar acquisition, contributes to pneumococcal adherence to the host epithelium and has been implicated as virulence determinant during bacterial transmigration from the lung and invasion of more distal sites. NanB and NanC have been characterized less extensively, and only a few studies have evaluated the roles of all three neuraminidases.

Monoclonal antibodies (mAbs) against pneumococcal neuraminidases were generated at Arsanis and used as tools to study the involvement of NanA versus NanB and/or NanC in lung infection models. Using primary human lung tissue, human lung cell lines and models of lethal murine pneumonia, we were able to assign individual roles of neuraminidases in pneumococcal interaction with human host cells (growth and adhesion) and demonstrate that NanA, but also NanB and/or NanC can desialylate human and murine lung tissues. Simultaneous targeting of neuraminidases was required to antagonize enzymatic function *in vitro* and to protect the terminal glycan structure of lung epithelial cells during murine pneumonia *in vivo*. However, in the model of severe murine pneumococcal pneumonia, no host benefit, such as survival, improved bacterial clearance or differences in inflammatory responses were observed. This is likely due to the model *per se*, which requires high bacterial challenge inocula and thereby circumvents nasopharyngeal colonization prior to aspiration of pneumococci to the lung.

Personal contributions:

Figure 1A, Figure 2A; performing the experiments for Figures 2B-6, S1, S2; Table S1-S3; neuraminidase gene deletion mutant generation; cytokine & chemokine measurements mentioned in the text (data not shown); fluorescence microscopy of stained lung tissues; data analysis, manuscript and figure preparation; Experiments involving infection of mice, tissue harvest and staining were not performed by myself.

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The manuscript entitled “Assessing the function of pneumococcal neuraminidases NanA, NanB and NanC in *in vitro* and *in vivo* lung infection models using monoclonal antibodies” was submitted to Virulence on April 11th, 2018. It is currently under revision (status May 18th, 2018). The confirmation note received from the journal including the manuscript number is shown below.

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Assessing the function of pneumococcal neuraminidases NanA, NanB and NanC in *in vitro* and *in vivo* lung infection models using monoclonal antibodies --Manuscript Draft--

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Abstract:	Streptococcus pneumoniae isolates express up to three neuraminidases (sialidases), NanA, NanB and NanC, all of which cleave the terminal sialic acid of glycan-structures that decorate host cell surfaces. Most research has focused on the role of NanA with limited investigations evaluating the roles of all three neuraminidases in host-pathogen interactions. We generated two highly potent monoclonal antibodies (mAbs), one that blocks the enzymatic activity of NanA and one cross-neutralizing NanB and NanC. Total neuraminidase activity of clinical <i>S. pneumoniae</i> isolates could be inhibited by this mAb combination in enzymatic assays. To detect desialylation of cell surfaces by pneumococcal neuraminidases, primary human tracheal/bronchial mucociliary epithelial tissues were infected with <i>S. pneumoniae</i> and stained with peanut lectin. Simultaneous targeting of the neuraminidases was required to prevent desialylation, suggesting that inhibition of NanA alone is not sufficient to preserve terminal lung glycans. Importantly, we also found that all three neuraminidases increased the interaction of <i>S. pneumoniae</i> with human airway epithelial cells. Lectin-staining of lung tissues of mice pre-treated with mAbs before intranasal challenge with <i>S. pneumoniae</i> confirmed that both anti-NanA and anti-NanBC mAbs were required to effectively block desialylation of the respiratory epithelium <i>in vivo</i>. Despite this, no effect on survival, reduction in pulmonary bacterial load, or significant changes in cytokine responses were observed. This suggests that neuraminidases have no pivotal role in this murine pneumonia model that is induced by high bacterial challenge inocula and does not progress from colonization as it happens in the human host.
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**Assessing the function of pneumococcal neuraminidases NanA, NanB and NanC in
in vitro and *in vivo* lung infection models using monoclonal antibodies**

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ABSTRACT

Streptococcus pneumoniae isolates express up to three neuraminidases (sialidases), NanA, NanB and NanC, all of which cleave the terminal sialic acid of glycan-structures that decorate host cell surfaces. Most research has focused on the role of NanA with limited investigations evaluating the roles of all three neuraminidases in host-pathogen interactions.

We generated two highly potent monoclonal antibodies (mAbs), one that blocks the enzymatic activity of NanA and one cross-neutralizing NanB and NanC. Total neuraminidase activity of clinical *S. pneumoniae* isolates could be inhibited by this mAb combination in enzymatic assays. To detect desialylation of cell surfaces by pneumococcal neuraminidases, primary human tracheal/bronchial mucocilial epithelial tissues were infected with *S. pneumoniae* and stained with peanut lectin. Simultaneous targeting of the neuraminidases was required to prevent desialylation, suggesting that inhibition of NanA alone is not sufficient to preserve terminal lung glycans. Importantly, we also found that all three neuraminidases increased the interaction of *S. pneumoniae* with human airway epithelial cells.

Lectin-staining of lung tissues of mice pre-treated with mAbs before intranasal challenge with *S. pneumoniae* confirmed that both anti-NanA and anti-NanBC mAbs were required to effectively block desialylation of the respiratory epithelium *in vivo*. Despite this, no effect on survival, reduction in pulmonary bacterial load, or significant changes in cytokine responses were observed. This suggests that neuraminidases have no pivotal role in this murine pneumonia model that is induced by high bacterial challenge inocula and does not progress from colonization as it happens in the human host.

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is one of the most common human pathogens. Transmitted by aerosols, *S. pneumoniae* colonizes the nasopharynx, the natural reservoir of this Gram-positive bacterium. From this site, bacteria can ascend to the sinuses (sinusitis) or the middle ear (otitis media). Pneumococcal spread to the lungs can cause pneumonia, followed by bacterial invasion into the bloodstream (bacteraemia) and ultimately penetration of the blood-brain barrier (meningitis), with disease severity increasing in this order [1]. The majority of deaths and the highest morbidities are generally observed among the elderly (> 65 years) and in children under the age of 5 years, especially in developing countries [2].

Based on the capsular polysaccharide composition, more than 90 pneumococcal serotypes have been identified. Vaccines have been developed that elicit immune responses against the most common capsular serotypes [1]. Since the introduction of these vaccines, a significant reduction in the rates of childhood pneumococcal disease has been observed. However, variable impacts of vaccination on invasive pneumococcal disease rates, such as bacteraemia, septicaemia or meningitis often combined with pneumonia, have been reported in the elderly [3,4]. Rates of non-bacteraemic pneumococcal pneumonia in this age group have remained largely unaffected [5,6]. Present challenges in combating *S. pneumoniae* infections include serotype replacement (i.e. strains covered by vaccines are being substituted by non-vaccine serotypes), different world-wide serotype distribution patterns, recently described emergence of pathogenic non-encapsulated strains and increasing antibiotic resistance [7–9]. Additionally, despite proper antibiotic treatment, mortality rates are high, especially in the elderly. This is thought to be caused, at least partly, by damaging inflammatory host responses induced by the pneumococcus. Therefore, anti-virulence approaches, such as those based on monoclonal antibodies (mAbs) targeting pneumococcal virulence factors are being considered to improve therapeutic efficacy [10–15].

Pneumococcal fitness relies heavily on the glycolytic metabolism [16]. However, the availability of free hexoses (such as glucose) in the healthy human respiratory tract is limited [17]. Therefore, different strategies have evolved in pneumococci to gain access to nutrient sources. One such mechanism is the utilization of sugars that decorate host cell surfaces, such as sialic acids. Neuraminidases (sialidases) are key enzymes in sugar acquisition. Three pneumococcal neuraminidases, NanA, NanB and NanC have been described, with reported gene frequencies of 100, 96 and 36-51 % among clinical isolates, respectively [18,19]. Neuraminidases share critical sequence features, such as the N-terminal signal peptide, a

carbohydrate binding module (CBM40) that recognizes sialic acid (lectin-binding domain), and a six-bladed β -propeller catalytic domain (sialidase domain) with an inserted domain [20,21]. NanA (115 kDa) additionally has a C-terminal LPETG membrane anchor motif, which is absent in NanB (78 kDa) and NanC (82 kDa). NanB shares approximately half of its amino-acid residues with NanC, but only one quarter with NanA. Differences in hydrophobicity around the active sites exist, whereas residues required for catalysis, such as a tri-arginine cluster that interacts with sialic acid, are conserved. All three neuraminidases recognize α 2,3-linked sialic acids, but only NanA shows considerable activity towards α 2,6-, and α 2,8-linkages [20–24].

NanA and NanB were shown to degrade terminal host glycoconjugates (desialylation), which enables other exoglycosidases to subsequently remove underlying sugars to provide key nutrients for the bacterium [25,26]. NanA is required for pneumococcal growth on mucins (sialylated glycoproteins abundantly found in the respiratory tract) [27] and its expression is upregulated in the presence of lung cells [28]. Moreover, sequential deglycosylation can expose otherwise hidden receptors, such as asialo-GM1, which was shown to act as a receptor for pneumococcal binding to the host epithelium [29–31]. Both NanA and NanB were demonstrated to be involved in colonization and infection of the upper and lower respiratory tract [32,33] and can contribute to biofilm formation [34–37].

Additionally, NanA was shown to play an important role during colonization and persistence in the nasopharynx and middle ear [38–40]. It is required for adherence and invasion of brain endothelial cells, indicating its involvement in the development of meningitis [41]. The role of NanA during sepsis is more controversial, with reports ranging from greatly attenuated virulence of *S. pneumoniae* *nanA* gene deletion strains to no effect [33,42].

Removal of host surface glycan-structures by NanA can induce pro-inflammatory responses, leading to recruitment of immune cells, which can be detrimental if uncontrolled [43].

The contribution of NanC to pneumococcal disease is not well understood. Although the presence of *nanC* has initially been suggested to be linked to *S. pneumoniae* isolates from cerebrospinal fluid [18], this finding could not be confirmed in a more recent study [44]. Pneumococcal isolates harbouring *nanC* have been implicated to be involved in necrotizing pneumonia and subsequent development of haemolytic uremic syndrome (HUS) [45].

NanA, NanB and NanC can cleave serum sialoglycoproteins, including fetuin-A, low levels of which were associated with invasive pneumococcal disease and HUS [46]. Notably,

immunization with all three recombinant neuraminidases was shown to modestly increase survival of mice challenged intravenously with different *S. pneumoniae* strains [47].

Given the multiple functions of neuraminidases in the pneumococcal life cycle and their complex interactions with the host, we hypothesized that inhibiting the activity of NanA, NanB and NanC by monoclonal antibodies could provide novel insights into the biological roles of these virulence factors and complement existing knowledge that has hitherto been generated mainly with recombinant neuraminidases or gene deletion mutant strains.

MATERIALS AND METHODS

Cloning, expression and purification of recombinant neuraminidases.

Published *S. pneumoniae* D39 NanA lectin domain (LD) and sialidase domain (SD) protein sequences (GenBank: ABJ55437.1, aa 16-279 and 280-756, respectively) were blasted against the NCBI database. A total of 310 unique protein sequences were analysed and for each domain, two clade variants were identified. Inter-clade sequence conservation was ~ 83 and 82 %, while intra-clade sequence conservation was > 94 and 96 % for the LD and SD, respectively. Three NanA recombinant proteins containing LD and SD from different clades (NanA-LD-SD, NanA-LDvar-SD, NanA-LD-SDvar) but missing the C-terminal hydrophobic region (downstream of the LPXTG anchor motif), and also NanB and NanC proteins (Table 1), were cloned in mature form (signal peptide predicted with SignalP 4.0 [48]), without tags, in pET24a, and overexpressed in *E. coli* in soluble form. Chromatographic protein purification was performed using anion exchange (HiTrap Q Sepharose FF, GE Healthcare) with a salt gradient, followed by anion exchange (HiTrap Q Sepharose HP, GE Healthcare) with a pH gradient and size exclusion (Superdex 200, 16/60, GE Healthcare). Purity and monomeric content for all proteins, both > 95%, were determined by SDS-PAGE, size exclusion chromatography and circular dichroism for the secondary structure. Enzymatic activities (k_{cat}/K_m) were determined using a fluorometric assay as described previously [49]. NanA baits for antibody library selections were generated by biotinylation using an amine reactive reagent (Sulfo-NHS-LC biotin, Pierce) according to manufacturer's instructions.

Pneumococcal strains, lysates and culture supernatants.

Clinical *S. pneumoniae* isolates collected in eight hospitals in Hungary between 2000 and 2008 from patients (aged between 8 months to 74 years) with invasive infections or

pneumonia [50] were kindly provided by Orsolya Dobay. The strains D39, A66.1 and EF3030 were kindly provided by David Briles; TIGR4 was obtained from ATCC (BAA-334™). Strain characteristics are outlined in Table S1.

Bacterial lysates were generated from cultures grown in THY medium (Todd Hewitt Broth + 0.5 % yeast extract; Sigma) to OD_{620nm} of 0.3 (37 °C, 5 % CO₂), pelleted, washed with PBS and homogenized using 2 mL tubes containing glass beads (Precellys® glass kit 0.1 mm) in a Precellys 24 tissue homogenizer. Total protein concentration of lysates was measured with a BCA kit (Pierce), according to the manufacturer's instructions.

Culture supernatants (CS) were generated from strains grown in THY medium to early stationary phase (37 °C, 5 % CO₂) as previously described [51]. Cultures were centrifuged (5.000 x g, 10 min, 4 °C), CS were harvested, sterile filtered (0.1 µm Millex® PVDF syringe-driven filter units, Millipore) and concentrated 15-fold (Vivaspin®20 centrifugal concentrators, MWCO = 10 kDa, Sartorius).

Generation of *S. pneumoniae* gene deletion mutant strains.

Isogenic *S. pneumoniae* gene deletion mutant strains (Δply , $\Delta nanA$, $\Delta nanB$, $\Delta nanA\Delta nanB$) were generated by allelic replacement in the D39 background as previously described [52], selecting for Ery^R or Kan^R clones on Columbia agar supplemented with 5 % sheep blood (COS, Biomerieux) and antibiotics (0.3 µg/ mL erythromycin, or 400 µg/ mL kanamycin). Primers used for mutagenesis are listed in Table S2. Competence in D39 was induced by CSP-1 (Eurogentec) in the presence of 1 % CaCl₂. Gene-deletions were verified by PCR and lack of functional activity (neuraminidase or pneumolysin). Growth rates of mutants in THY medium (37 °C, 5 % CO₂) did not differ from the D39 parental strain.

DNA isolation, neuraminidase gene screening and genotyping of clinical *S. pneumoniae* isolates.

Pneumococcal genomic DNA was isolated using the DNeasy Blood and Tissue Kit 250 (Qiagen) according to the manufacturer's instructions. Detection of *nanA*, *nanB* and *nanC* gene fragments (~ 500 bp) was performed by PCR as described previously [18]. Neuraminidase genes (~ 3000 bp) were amplified using primers 31R and 31S [53] (*nanA*), B_seq1 and B_seq8 (*nanB*), or C_seq1 and C_seq8 (*nanC*); genes encoding pneumolysin (Ply) and PspA clade defining regions as described elsewhere [54–56]. Amplicons were sequenced using primers listed in Table S3. Sequences were assembled and aligned using the CLC Main Workbench 6 software (Qiagen).

Generation of anti-NanA mAbs.

NanA-specific mAbs were generated by *in vitro* selection using full length human IgG1 libraries expressed by yeast as previously published [57–60] and biotinylated NanA-variants as baits. Antibody clones were characterized as described below and selected for affinity-maturation (light chain diversification and subsequent heavy chain mutagenesis).

VH and Vk sequences of selected mAbs were cloned into pTT5 mammalian expression vectors (Biotechnology Research Institute, National Research Council of Canada, Quebec) in-frame with human IgG1/ human kappa light chain constant regions. MAb were expressed in CHO-3E7 cells (Biotechnology Research Institute, National Research Council of Canada, Quebec) by co-transfecting with pTT5 plasmids encoding heavy and light chains using PEI MAX (Polysciences) for DNA delivery. MAb were purified from supernatants 8 days post transfection using HiTrap MabSelect or MabSelectSuRe (for the mAbs of human VH3 germline) pre-packed columns (GE Healthcare).

Generation of anti-NanBC cross-reactive mAbs.

Mice were subcutaneously immunized three times with recombinant NanB (10 µg/ mouse/ immunization, formulated with Alum or Freund's adjuvant) and hyperimmune sera were tested for cross-reactivity against NanC. The mouse with the highest cross-neutralizing titer was intravenously boosted with NanC (5 µg) for subsequent hybridoma generation according to standard protocols.

Selected hybridoma clones expressing mAbs that neutralized both enzymes were subjected to sequencing of antibody heavy and light chain genes. Briefly, mRNAs isolated from these clones were reverse-transcribed. MAb VH and Vk sequences were amplified from the respective cDNAs using heavy and light chain primer mixes (GE Healthcare) and cloned into a pJET1.2 vector (Fermentas) for sequencing with vector-specific primers. Sequences were analyzed against the mouse VH and Vk germline repertoire with IMGT V-QUEST [61] to determine germline type, presence of somatic mutations, position and length of the CDRs and framework regions.

To generate chimeric mAbs, the identified mouse VH and Vk were fused with human IgG1/ human kappa light chain constant regions in a pTT5 vector. MAb were produced in CHO-3E7 cells as described above.

Binding affinity determination of mAbs.

For the affinity determination of α -NanA mAbs, binding of the Fab fragments to NanA and its sequence variants was measured by biolayer interferometry using a ForteBio Octet Red Instrument (Pall Life Sciences). The biotinylated antigen was immobilized on the streptavidin sensor and the association and dissociation of the Fab fragment (100 nM), in solution (PBS, pH 7.2 + 1 % BSA), were monitored for 3 min each. The monovalent affinities expressed as dissociation constants (K_D values) were calculated based on the measured kinetic parameters (k_{on} and k_{off}) by simultaneously fitting the association and dissociation phases to a 1:1 binding model using Octet Data Analysis Software version 7. In an alternative setup the mAb was immobilized on AHC sensor and the association and dissociation of the antigen (200 nM for NanA-SD or NanA-LD), in solution (PBS, pH 7.5 + 0.1 % BSA), at 30 °C, were monitored for 5 and 3 min, respectively. Affinities of α -NanBC mAbs for NanB and NanC were also measured by biolayer interferometry: the chimeric mAb was immobilized on the anti-human capture sensor and the association and dissociation of the antigen (100 nM), in solution (PBS, pH 7.5 + 0.1 % BSA), at 30 °C, were monitored for 10 min each. Monovalent affinities and kinetic parameters were determined as for NanA.

***In vitro* neuraminidase enzymatic assay.**

Neuraminidase activity was measured in a fluorometric assay using the sialic acid analogue 2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic acid sodium salt hydrate (NEU5AC-A-4MU, Sigma) as substrate [49] in HBSS + 0.5 % BSA buffer. Fluorescence (λ_{ex} = 365 nm, λ_{em} = 450 nm) was detected using a BioTek-Synergy HT plate reader.

Assay conditions were adapted to measure neuraminidase activity of recombinant neuraminidases, pneumococcal lysates or CS in the presence of α -neuraminidase mAbs as specified in the corresponding figure legends. Data were collected at 5-30 min, 4 h, or 10 h after addition of the substrate (200- 400 μ M), respectively.

Human cell surface desialylation assay.

Raji cells (ATCC® CCL86™) used at 2.5 to 5 x 10⁵ cells/ well were incubated with recombinant NanA (1 nM) at 4 °C for 30 min or infected with *S. pneumoniae* D39 Δ ply at a multiplicity of infection (MOI) 10 for 3 h at 37 °C in RPMI (Gibco, Thermo Fisher Scientific) + 10 % FCS (Sigma). Inhibition of cell surface desialylation by α -NanA mAbs (4 - 1000 nM) was measured by flow cytometry (BD Accuri) using anti-asialo-GM1 polyclonal rabbit IgG

(eBioscience) and Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG H+L (Molecular Probes) as secondary reagent, or an *A. hypogaea* (peanut) lectin FITC conjugate (Sigma).

***In vitro* infection assay using the A549 lung epithelial cell line.**

S. pneumoniae strains were grown in THY medium to OD_{620nm} of 0.3, pelleted, washed with PBS and re-suspended in antibiotic-free F12K medium (Invitrogen) + 10 % FCS (Sigma). A human pulmonary epithelial cell line (A549; ATCC® CCL-185™; adenocarcinomic alveolar basal epithelial cells), grown to confluent monolayers in the same medium, was infected at MOI 10 for 48 h (for D39 and mutants), 3 or 24 h (for EF3030 and Sp#5) at 37 °C, 5 % CO₂.

At the end of infection supernatants were harvested (to collect unbound bacteria), A549 cells were washed twice with PBS, detached and lysed using Trypsin/ EDTA (Invitrogen) and 2 % saponin (Sigma), respectively. Serial dilutions of bacteria in the supernatant and those recovered from A549 cells were plated onto Columbia agar plates supplemented with 5 % sheep blood (COS, Biomerieux) and incubated overnight (37 °C, 5 % CO₂) for CFU enumeration.

Human 3D mucociliary lung tissue model.

Multi-layered, mucus-producing normal human tracheal/ bronchial lung epithelial cells (EpiAirway™, MatTek 24 or 96 well) were processed according to the supplier's protocol.

Pneumococcal strains were prepared as above and re-suspended in antibiotic-free medium (MatTek). To study desialylation, tissues were infected with D39Δply (MOI 100) or treated with recombinant neuraminidases. Alternatively, tissues were infected with *S. pneumoniae* EF3030 or Sp#5 at MOI 5 in the presence of α-neuraminidase and α-Ply mAbs. Tissues were incubated for 24 h (37 °C, 5 % CO₂) and subsequently fixed (3 h at room temperature) with 10 % neutral buffered formalin solution (Sigma) for fluorescence microscopy.

Additionally, the bacterial interaction with the human lung tissue was evaluated from experiments with the EF3030 or Sp#5 strains. Supernatants of infected samples were collected at the end of the incubation time (to harvest unbound bacteria), tissues were washed twice with PBS, removed using sterile tissue punches (3.5 mm, Integra, Miltex), homogenized with a Precellys 24 homogenizer (Precellys bulk beads 1.4 mm Zirconium oxide) and treated with 2 % saponin (Sigma) for subsequent CFU enumeration as described above.

Murine *S. pneumoniae* pneumonia models.

Animal experiments were performed according to Austrian Law (BGBl. I Nr. 114/2012, approved by MA58, Vienna, Austria) and IACUC approval, with groups of 5 female, 6-8 week-old C57BL/6JRj mice (Janvier, France). MAbs (200-300 µg/ mAb/ mouse, formulated in PBS) were given by intraperitoneal injection, 48 h prior to intranasal bacterial challenge. Control groups received an isotype-matched (human IgG1) control mAb. Mice were anesthetized with ketamine/xylazine followed by drop-wise intranasal application of the bacterial challenge inoculum ($2-3 \times 10^7$ CFU/mouse). Survival was monitored for up to 10 days post challenge or animals were sacrificed after 24 or 48 h for the determination of bacterial organ loads (enumeration of bacterial CFUs in serial fold-dilutions of the respective organ after plating on COS agar plates and incubation at 37 °C, 5 % CO₂, > 16 h) or for staining of lung tissues. Statistical analyses were performed with the log-rank (Mantel-Cox) test using GraphPad Prism 6 software.

Tissue staining to detect desialylation.

Formalin-fixed lung tissues and mouse lungs were embedded in paraffin to generate histological sections of 3 µm, or 1 µm thickness respectively. Samples were stained with *A. hypogaea* (peanut) lectin-FITC conjugate (10 µg/ mL) to detect desialylation (Sigma) and Hoechst 33258 (Sigma) to stain cell nuclei. Samples were visualized with an Olympus VS120 S6-E microscope (VS-XM10tR camera, UPLSAPO40x objective NA=0.95) and analysed with OlyVIA 2.9 software. Background was removed without obscuring biological information.

Determination of cytokine and chemokine levels in lung tissue.

Lungs of mice infected with *S. pneumoniae* EF3030 or Sp#5 in presence of α-neuraminidase or control mAbs were excised, washed in PBS and weighed before homogenization (Precellys 24 homogenizer, Precellys bulk beads 1.4 mm Zirconium oxide). Proteinase inhibitor cocktail (Sigma) was added to the homogenized samples, which were centrifuged (16.000 x g, 15 min, 4 °C) to harvest supernatants (stored at -80 °C until use).

Cytokine and chemokine levels in supernatants were determined using the Mouse Inflammation Panel and Mouse Proinflammatory Chemokine Panel (LEGENDplex™ multiplex cytokine bead arrays; BioLegend) according to the manufacturer's instructions. Samples were measured on a CytoFLEX flow cytometer (Beckman Coulter) and results were analysed using LEGENDPlex 7.0 software (BioLegend). Analyte levels were normalized to the weights of lung tissues.

RESULTS

Generation of α -NanA mAbs to inhibit the activity of different NanA variants.

NanA contains two main functional domains, the lectin-binding domain (LD) and sialidase domain (SD). Amino acid sequence alignment of publicly available NanA sequences (n= 310) allowed clustering into three main groups: NanA-LD-SD (represented by strain D39), NanA-LDvar-SD (variant LD domain, but SD conserved with prototype strain D39, example strain 70585) and NanA-LD-SDvar (D39 like LD domain with variant lectin domain, example strain INV200). These three variants represented 74, 5 and 13 % of published NanA sequences, respectively, and cover > 90 % of NanA diversity. This grouping therefore also provided the basis for antigen expression and all three bait types were used for antibody selection. The highest NanA amino acid sequence diversity was observed in the regions around the sugar binding site of the LD and the insertion sequence of the SD (Fig. 1A).

To cover a broad spectrum of pneumococcal diversity and to exclude strain-specific effects during mAb generation, we chose 20 highly diverse clinical *S. pneumoniae* isolates and four reference strains (D39, A66.1, EF3030 and TIGR4) based on serotype, PspA clade and *ply* allele (strain characteristics are shown in Table S1). Gene frequencies for *nanA*, *nanB* and *nanC* were 100, 96 and 38 %, respectively among these strains.

The overall amino acid identities of NanA in this set of strains (without the signal peptide) was determined to be ≥ 83 % (excluding TIGR4, which encodes a truncated NanA). Three NanA clusters could be identified in line with the representative variants from published sequences (Fig. S1). The overall mean distance (Poisson model, Gamma distribution) between the three NanA clusters was 0.066 as opposed to the within group distances of 0.012 (cluster 1), 0.009 (cluster 2), or 0.005 (cluster 3). No variations of amino acid residues previously determined as essential for enzymatic activity were identified in these strains [63].

Using yeast expression libraries of human IgG1 sequences, we selected approximately 80 mAbs binding to either the SD or LD based on biolayer interferometry measurements and immunoblotting with the respective recombinant domains. Although only the SD-binding mAb fraction showed inhibitory activity of NanA with a synthetic substrate in the fluorescence assays (Fig. 1B), both SD- and LD-binding mAbs were able to block desialylation of eukaryotic cells (Fig. 1C), indicating activity against the natural substrate for both mAb types. The most potent “naïve” mAbs were subjected to affinity maturation with light chain shuffled and

HCDR1 and HCDR2 (H1H2) mutated sub-libraries. Next, we selected mAbs with high inhibitory potential and broad reactivity against NanA sequence variants, tested with the panel of pre-characterized *S. pneumoniae* isolates (Fig. 1D). Based on the cumulative inhibition of NanA activity of these strains, as well as highest neutralizing potency against the major NanA variant (NanA-LD-SD) upon infection of Raji cells with *S. pneumoniae* D39 Δ ply (to avoid cell lysis) (Fig. 1E), mAb #21155, a SD-binder, was selected for further studies. Binding affinities of this mAb to NanA-LD-SD, NanA-LDvar-SD and NanA-LD-SDvar were in the low nanomolar range (Table S4). No cross-reactivity of this mAb to NanB or NanC could be detected.

Generation of cross-reactive α -NanBC mAbs.

Sequencing of *nanB* and *nanC* from the 24 pre-selected *S. pneumoniae* strains (Table S1) revealed ≥ 97 % conservation at the amino acid level for both NanB and NanC. Protein identities between NanB and NanC were approximately 46 %, and to NanA (without C-terminal anchor) approximately 20 %. The majority of the few conserved residues between NanA and the other two neuraminidases were located around the sugar binding site of the SD. In contrast, NanB and NanC showed high sequence conservation around the sugar binding sites of both the SD and the LD, as well as the region opposite to the SD active center (Fig. 2A).

We aimed at generating cross-reactive α -NanBC mAbs using a murine hybridoma approach employing alternating immunization of mice with both antigens. Mixed clone hybridoma supernatants (n= 1920) were tested for binding to NanB and NanC (by ELISA), followed by screening for neutralization of enzymatic activities using recombinant proteins (Fig. 2B). Several clones showed cross-reactivity and cross-neutralization in the single clone stage and the five most potent hybridoma clones were subjected to DNA sequencing. Three of them had unique VH and Vk sequences and were expressed as mouse-human chimeric antibodies. Based on its highest *in vitro* potency, mAb 3C6-H7 was selected for further studies (Fig. 2C). Binding affinities of this mAb to NanB and NanC were in the picomolar range (Table S4). No cross-reactivity of 3C6-H7 to NanA could be detected.

Contribution of the three neuraminidases to the total neuraminidase activity varies substantially among different *S. pneumoniae* isolates.

The activity and contribution of NanA vs. NanB and/or NanC to the total pneumococcal neuraminidase activity was addressed in the enzymatic fluorescent assay using pneumococcal early stationary phase culture supernatants (CS) of six *S. pneumoniae* strains in the presence of

the α -NanA and α -NanBC mAbs (Fig. 3). Since NanB and NanC are secreted proteins, these experiments were performed with CS, where NanA is also present (despite being mainly surface anchored, except for the TIGR4 strain). This *in vitro* model was validated by showing complete inhibition of the enzymatic activity of an equimolar mixture of the three recombinant neuraminidases (200 pM each) by α -NanA and α -NanBC mAbs (1 μ M each) (Fig. 3A). Importantly, the neuraminidase activities of the CSs at the tested dilution were lower in this assay compared to those of the recombinant proteins inhibited with the same amount of mAbs.

We found that inhibition of NanA alone was sufficient to abolish enzymatic activity of some strains (D39, TIGR4). Blocking NanB and/or NanC was required to fully inhibit the neuraminidase activity of another subset of strains (EF3030, Sp#150). Neither mAb alone could inhibit neuraminidase activity of a third type of strain (Sp#5), however, the combination of both mAbs led to full inhibition. Neuraminidase activity of one strain (Sp#36) was successfully inhibited by the α -NanBC mAb alone (Fig. 3B), suggesting lack of any NanA activity. Indeed, sequence analysis revealed a frameshift mutation resulting in a truncated NanA protein (only 74 amino acids long). To our knowledge this is the first reported naturally occurring *S. pneumoniae* strain that does not produce functional NanA.

These data suggest that the total neuraminidase activity in different *S. pneumoniae* isolates is highly variable and can involve one or up to all three neuraminidases. Efficient and complete inhibition of enzymatic sialidase activity in clinical *S. pneumoniae* isolates therefore necessitates simultaneous targeting of all three neuraminidases.

Pneumococcal neuraminidases desialylate human lung tissue *in vitro*.

To mimic a relevant *in vivo*-like situation, we employed an *in vitro* lung infection model, in which a human, three-dimensional multi-layered and mucociliated airway epithelial primary tissue was infected with *S. pneumoniae*. The activity of the *in situ* produced neuraminidases was tested by detecting cellular desialylation, and their contributions were dissected by neutralization with the α -NanA and/or α -NanBC mAbs. We used a staining method that employed fluorescently labelled peanut lectin which specifically recognizes the sugar moiety Gal β 3GalNAc that only becomes unmasked after neuraminidase-mediated removal of terminal sialic acids. First, we confirmed that each individual recombinant neuraminidase was able to desialylate human lung airway epithelial cells in this tissue model (data not shown). Desialylation by an equimolar mixture of all three recombinant neuraminidases was greatly reduced in the presence of the mAb-combination but only partially by the individual mAbs (Fig. 4A). In agreement with this, prevention of desialylation during

infection with *S. pneumoniae* D39 Δ ply required both α -neuraminidase mAbs (Fig. 4B). Similar results were obtained with two other *S. pneumoniae* strains, EF3030 or Sp#5 (data not shown).

These data suggest that pneumococcal neuraminidases are active towards the human airway epithelium and that α -neuraminidase mAbs can preserve the terminal epithelial sugar composition during *S. pneumoniae* infection.

Pneumococcal neuraminidases enhance *S. pneumoniae* interaction with human epithelial cells.

We further evaluated the contribution of individual pneumococcal neuraminidases to the interaction of *S. pneumoniae* with lung cells in several *in vitro* infection assays.

Monolayers of human lung alveolar cells (A549) were infected with *S. pneumoniae* D39 (*nanC* negative) or isogenic neuraminidase gene deletion mutants (Fig. 5A, left panel) or wild-type D39 in presence of the α -NanA and α -NanBC mAbs (Fig. 5A, middle panel). Removal of NanA (either by gene deletion or neutralization with the α -NanA mAb) reduced the number of bacteria recovered from A549 cells by approximately 10-fold, while removal of NanB had minimal effects. However, lack of both NanA and NanB reduced the recovered CFUs approximately 30 to 100-fold.

Reciprocally, D39 Δ *nanA* Δ *nanB* was used to infect A549 cells in presence of recombinant neuraminidases (Fig. 5A right panel). Addition of NanA increased the number of bacteria recovered from A549 cells by approximately 25-fold, whereas the addition of either NanB or NanC resulted in approximately 10-fold increased CFUs. Importantly, the presence of both NanB and NanC resulted in similar numbers of recovered bacteria as NanA alone. Addition of NanB or NanC on top of NanA did not enhance this effect, however, the presence of all three neuraminidases resulted in approximately 70-fold more bacteria associated with A549 cells. Samples treated with D39 wild-type and control protein showed similar CFUs as those infected with D39 Δ *nanA* Δ *nanB* and treated with NanA, or NanA and a second neuraminidase.

Using two additional *S. pneumoniae* strains, EF3030 (*nanA/B*) and Sp#5 (*nanA/B/C*) in the same assay, more pronounced differences upon mAb-treatment were observed. Neutralization of NanB (EF3030), or neutralization of NanB and/or NanC (Sp#5) reduced bacterial numbers to the same level as seen with neutralization of NanA in a time-dependent fashion (Fig. 5B). Simultaneous targeting of all neuraminidases further enhanced this effect, resulting in up to 3-log CFU reduction relative to the control group after 24 h.

In the multi-layered primary human airway epithelial tissue model, blocking of the neuraminidases by the two mAbs during EF3030 and Sp#5 infection resulted in highly similar effects on bacterial recovery (Fig. 5C), although this was less pronounced (approximately 10 to 20-fold decrease).

In the same models, we also assessed the effect of neuraminidases on pneumococcal growth by CFU plating of bacteria recovered from supernatants. When A549 cells were infected for ≥ 24 h, the absence of neuraminidases resulted in up to 3 to 4-log reductions in bacterial growth and resembled changes observed with bacteria recovered from host cells. However, bacterial growth in the supernatants of A549 cells infected for 3 h, or primary human lung tissue infected for 24 h did not differ between experimental groups (data not shown).

These data suggest that all three neuraminidases enhance the interaction of pneumococci with human lung cells *in vitro* by increasing growth, or direct contact with host cells, or both.

Pneumococcal neuraminidases desialylate murine lungs during *S. pneumoniae* infection *in vivo*.

In order to investigate neuraminidase-mediated lung desialylation *in vivo*, mice were challenged intranasally with *S. pneumoniae* D39 wild-type (*nanA/B*) or D39 Δ *nanA* Δ *nanB*. We observed strong desialylation of the conductive zones (comprising main bronchi and terminal bronchioles) and respiratory zones (respiratory bronchioles, alveolar areas, interstitial cells) upon challenge with the wild-type strain, but not with the neuraminidase-deficient strain (Fig. 6A). Importantly, simultaneous neutralization of NanA and NanB by prophylactic administration of both α -neuraminidase mAbs abolished *S. pneumoniae* D39-mediated pulmonary desialylation (Fig. 6B).

The contribution of NanA and NanB and/or NanC to desialylation was further assessed in experiments where mice were treated with α -NanA and α -NanBC mAbs and infected with strains EF3030 (*nanA/B*) or Sp#5 (*nanA/B/C*). Neutralization of NanA slightly reduced desialylation of the pulmonary conducting zone and further reduced desialylation of the respiratory zone in mice infected with EF3030 for 24 h. In the Sp#5 infection model, targeting NanA alone did not affect desialylation 24 h after infection. Neutralization of NanB and/or NanC did not have detectable effects in either model. However, antagonizing all neuraminidases with the α -NanA and α -NanBC mAbs led to greatly reduced desialylation of the conducting and the respiratory zones with a more pronounced effect seen in the EF3030 model (Fig. 6C, Fig. S2). Infection with Sp#5 for 48 h showed similar results as seen in the

EF3030 model at 24 h, however, a relatively greater effect of NanB and/or NanC was observed in the Sp#5 model. Interestingly, desialylation in the respiratory zones was only detected if the conducting airways of the same lung were also affected, but not *vice versa*.

These data show that pneumococcal neuraminidases greatly affect the lung glycan-structure in acute murine pneumonia models. NanB and/or NanC can potentially desialylate the murine lung epithelium in the absence of NanA.

Neutralization of pneumococcal neuraminidases during acute murine pneumonia does not affect survival, lung bacterial burden, or host inflammatory responses.

Upon detecting the pronounced inhibitory effects of the α -neuraminidase mAbs on desialylation of the lung epithelium by *S. pneumoniae*, we tested their efficacy on survival, bacterial organ load and inflammatory responses in mice.

We found that animals pre-treated with α -NanA or α -NanBC or both mAbs 48 h before challenge with lethal doses of *S. pneumoniae* strains EF3030 or D39 did not show improved survival compared to control animals (Fig. S3A). Bacterial burden in the lungs was also measured in control and mAb-treated animals at 24 h post-infection with *S. pneumoniae* strains EF3030 or Sp#5. Again, no beneficial effect was observed (Fig. S3B). Since cellular desialylation has been implicated as a pro-inflammatory signal, we determined the pulmonary levels of key cytokines and chemokines (including TNF- α , IL-1 β , IL-6, interferons, KC, MIPs, MCP-1 and CXCR3 ligands) in the same animals used for the determination of the bacterial lung burden. Despite clear induction of inflammatory host responses by *S. pneumoniae*, no significant changes were seen in mice treated with α -neuraminidase mAbs (data not shown).

These data suggest that neuraminidases, although active *in vivo*, do not play a pivotal role in this murine model of acute pneumonia.

DISCUSSION

Pneumococcal neuraminidases have been implicated as virulence determinants, mainly based on experiments using gene deletion mutants of laboratory strains of *S. pneumoniae*, both *in vitro* and *in vivo*. Previous studies reported a substantially reduced bacterial burden in the lungs of mice infected intranasally with *nanA* and/or *nanB* gene deletion mutant strains and improved host survival [32,33,64]. NanA was shown to contribute to nasopharyngeal colonisation [37,38,40,64,65]. However, these findings contrast to several other publications,

where no such effects were observed *in vivo* [25,51,66–68]. Apart from differences in the bacterial strains and challenge doses, the reliability of generating true isogenic mutant strains may be a concern, especially when using a bacterium like the pneumococcus that is notorious for its genomic instability.

We generated two monoclonal antibodies that neutralize NanA, and NanB and NanC with high efficiency. This enabled us to characterize the involvement of the neuraminidases in *S. pneumoniae* lung infection models without potential artefacts associated with gene deletion mutant strains. The α -NanA mAb used in this study (#21155) was carefully selected to inhibit the activity of all major variants of NanA. A recent study described three representative NanA sequences (Genbank KJ850445.1, KT893379.1, KT893380.1) with distinct biochemical properties and susceptibilities to inhibitors [69]. These correspond to the clusters 1, 2, and 3, respectively, in this study. Importantly, all variants are equally recognized by mAb #21155. The other α -neuraminidase mAb used in this study (3C6-H7) neutralizes both NanB and NanC with comparably high potency.

Recently, compounds have been identified that can impair pneumococcal neuraminidase activity *in vitro* based on enzymatic or functional inhibition assays [70,71]. Our study extends these findings by demonstrating antibody-mediated inhibition of the neuraminidases during infection of human lung tissue and in mouse pneumonia models.

Neuraminidase activity of different pneumococcal isolates can differ greatly [27], for various reasons, such as the presence of one to three different neuraminidase genes [18] and NanA mosaicism that can influence enzyme activity [69]. Regardless of the underlying molecular mechanism, our study provides evidence that neuraminidase activity across a range of different pneumococcal isolates can be antagonized by the combination of α -NanA and α -NanBC mAbs *in vitro* and *in vivo*.

NanA and NanB have been shown to be involved in pneumococcal adhesion to epithelial cells and colonization of the host [34–37]. In this study, we investigated the contribution of the three neuraminidases to the interaction with the host. We did this by determining bacterial numbers recovered from host cells and from the supernatant after infection. We demonstrate that the presence of recombinant NanA and NanB, but importantly also NanC, increased the number of bacteria recovered from A549 lung epithelial cells during infection with a neuraminidase-deficient *S. pneumoniae* strain. On the other hand, in the absence of neuraminidase function, we detected reduced interaction of three different *S. pneumoniae* strains with epithelial cells. In these models, bacterial growth in the supernatant was affected if longer infection times (≥ 24 h) were used, but not at 3 h post-infection.

Therefore, the increased bacterial numbers recovered from A549 cells at the early stages of infection likely arose from direct effects of the neuraminidases on bacterial interaction with the host cells (such as unveiling receptors to facilitate bacterial binding). At later stages, these differences were at least partly mediated by growth rates. MAb-based targeting of neuraminidases presumably leads to limited availability of sialic acid which can result in reduced growth, as shown before [35].

To more closely mimic the human lung environment *in vitro*, we used a human multilayered bronchial/epithelial 3D tissue infection model. In line with the results obtained with A549 cells, mAb-based inhibition of either NanA or NanB/C during pneumococcal infection reduced bacterial numbers recovered from primary human lung tissues 24 h post infection. Simultaneous targeting of all neuraminidases greatly enhanced this effect and protected the epithelial glycan-structure. In this model, bacterial growth in the supernatant was not influenced by the activity of the neuraminidases, therefore differences in bacterial numbers recovered from lung tissue potentially originated from direct effects at the lung cell surface.

The observed differences in the number of recovered bacteria in the absence of neuraminidases was greater with A549 cells compared to the 3D tissue culture model (up to 3 vs. 1 log), which is likely explained by the different effect on growth. This might be due to different amounts of, or access to sialylated glycan-structures. The malignant nature of A549 cells is known to affect surface glycosylation. For example, sialic acid molecules cluster differently on the A549 cell surface compared to human bronchial epithelial cells [72]. Moreover, A549 cells are not multi-layered and do not produce mucus. This might eliminate an important source of sialic acid used for growth during infection [27]. However, in the absence of this major natural barrier the pneumococcus may more readily access underlying sugars and adhere to host cells.

In murine pneumonia models, we demonstrate that targeting of all neuraminidases is required to protect the terminal epithelial pulmonary glycan-structure. MAb-based inhibition of neuraminidase-mediated desialylation of the respiratory zones (such as the alveolar areas with resident pulmonary and immigrating immune cells) was more readily observed than inhibition of desialylation of the conducting zones (such as the main bronchi and terminal bronchioles). This likely depends on bacterial density and spread. To gain more insight into desialylation kinetics, we infected mice with a *S. pneumoniae* strain (Sp#5) that carries all three neuraminidase genes (*nanA/B/C*). In spite of the inability of Sp#5 to induce lethal pneumonia in mice and its almost complete clearance from the lungs after 48 h of infection, desialylation was still clearly detectable at this time point. This suggests that bacterial clearance can be faster

(host mediated in our model or due to antibiotics) than re-sialylation of pulmonary surface glycans.

Despite the observed efficient inhibition of *S. pneumoniae*-induced desialylation of murine lung tissues, we could not detect increased survival or a reduction in bacterial burden in the lungs of infected animals. This suggests that preservation of the terminal lung epithelial glycosylation pattern does not improve host defences in this murine model of lethal pneumonia. In this model, intranasal instillation of considerably high minimally-lethal bacterial challenge doses was required for each strain. An inherent limitation of this model is that a major step in the natural progression towards pneumonia, namely nasopharyngeal colonisation followed by bacterial translocation into the lower airways, cannot be recapitulated. It has been previously suggested that once sufficient numbers of bacteria are present in the murine lung, the pneumococcus employs virulence mechanisms to survive and further invade distal sites that do not involve NanA [32]. Our study supports this notion and extends this observation to the other neuraminidases (NanB and/or NanC). The importance of neuraminidases in bacterial growth by acquisition of host sugars might greatly differ in a non-inflamed compared to an inflamed organ, as the latter contains substantially higher levels of free glucose [17], which renders *S. pneumoniae* less dependent on sialic acid. It is noteworthy that mouse strain-dependent differences in the sialic acid composition have been reported [73]. Importantly, human and mouse airways differ in their pulmonary sialic acid repertoire [74,75] and the pneumococcus has evolved to specifically sense and respond to human sialic composition [76]. Therefore, the role of neuraminidases in human pneumonia is potentially underestimated by extrapolating from mouse models.

NanA was reported to increase pulmonary levels of pro-inflammatory cytokines by desialylating Siglec receptors on cell surfaces, which disrupts inhibitory signalling [43]. Pre-treatment of mice with the α -neuraminidase mAbs, and the proven neutralization of the neuraminidases could not reduce lung inflammation based on the lack of effect on cytokine and chemokine levels. It is likely that in such a stringent model with high bacterial inocula, the absence of a colonization phase and the multilobar nature of infection, more direct mechanisms such as pneumolysin-mediated killing of host cells drive the pathogenesis of pneumococcal pneumonia. This is underlined by the findings that administration of α -pneumolysin mAbs [11] or induction of serum antibodies by vaccination with recombinant pneumolysin [10] protects mice from lethal pneumococcal pneumonia.

In summary, this study demonstrates that NanA, NanB and NanC can remove terminal sialic acids from human lung tissue and confer an advantage to *S. pneumoniae* during its

interaction with human lung cells *in vitro*. Antibody-based targeting of neuraminidases can greatly reduce neuraminidase activity *in vitro* and can also protect the murine lung epithelium from desialylation during acute pneumonia *in vivo*. However, we could not provide evidence for a decisive role of the neuraminidases in the acute phase of severe pneumonia in the mouse model applied. Non-invasive animal models, such as upper airway colonization and transmission to the lower airways, as well as otitis media might be more relevant models to further study the roles of neuraminidases during *S. pneumoniae* infections.

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Conflict of interest

The authors declare a potential conflict of interest as they are employees of Arsanis Biosciences GmbH (Vienna, Austria) and shareholders in Arsanis, Inc (USA), a biotechnology company developing monoclonal antibody-based products targeting bacterial infections.

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FIGURE LEGENDS

Figure 1. Generation of α -NanA mAbs. **A:** Sialidase (blue, PDB 3H72 [22]) and lectin (red, PDB 4C1X [62]) domains of NanA aligned (using PyMOL software v2.0.5; Schrödinger) on the NanB structure (PDB 2VW1 [21]) as a scaffold. Residues differing from NanA-LD-SD are highlighted in green, and sugars occupying the sugar binding sites are shown in yellow. **B:** Inhibition of neuraminidase activity of recombinant NanA (2.5 nM) and NanA from D39 lysates (used at a total protein concentration of 20 μ g/ mL) by naïve yeast library derived α -NanA mAbs (1 or 0.33 μ M, respectively) in a fluorometric assay. **C:** Inhibition of 1 nM rNanA-mediated Raji cell surface desialylation by naïve α -NanA mAbs (1 μ M). **D:** Cumulative inhibition of NanA sialidase activity in pneumococcal lysates (20-40 μ g/ mL) by selected naïve, and LCS (light chain shuffled) or mutated HCDR1 + HCDR2 (H1H2) SD-binding mAbs (0.33 μ M) in a fluorometric assay. Inhibition scores for each lysate from 0 (no inhibition) to 1 (100 % inhibition) were calculated relative to a control human IgG1. Cumulative inhibition scores were generated by addition of individual values (minimum score= 0, maximum score= 22). **E:** Inhibition of Raji cell surface desialylation during infection with *S. pneumoniae* (D39 Δ ply) by H1H2 α -NanA mAbs at the indicated concentrations.

Figure 2. Discovery of cross-reactive α -NanBC mAbs. **A:** Conservation among NanA, NanB and NanC. NanA structure as depicted in Figure 1, with residues identical in NanB (left panel) and NanC (4YC4 [23], middle panel) marked in black, and NanB (PDB 2VW1 [21], right panel, colors as for NanA) with residues identical in NanC marked in black. **B:** Mouse hybridoma mixed clone supernatants tested for neutralization of recombinant NanB and NanC (2 nM each) in a fluorometric assay. **C:** Three human-mouse chimeric mAbs expressed in CHO cells, tested at the indicated concentrations for inhibition of NanB and NanC.

Figure 3. Contribution of individual Nans to the total neuraminidase enzymatic activity differs among *S. pneumoniae* strains. Neuraminidase activity of recombinant neuraminidases (200 pM each) (**A**) or pneumococcal stationary phase CSs (**B**) in the presence of α -neuraminidase mAbs (1 μ M each). Two clones per strain were used for CS generation. Percent inhibition was calculated relative to a negative control mAb. Results are shown as mean with standard deviation.

Figure 4. Multiple neuraminidases contribute to desialylation of primary human lung tissue by *S. pneumoniae*. Primary human lung tissue treated with an equimolar mixture of all three recombinant neuraminidases (5 nM each) +/- α -neuraminidase mAbs (2 μ M each) (**A**) or infected with *S. pneumoniae* D39 Δ ply +/- α -neuraminidase mAbs (2 μ M each) or control mAb (4 μ M) (**B**) for 24 h. Desialylation was visualized by Peanut Lectin-FITC staining (green). Nuclei were stained with Hoechst dye (blue). Representative images of one experiment with biological duplicates are shown. Scale bar= 50 μ m.

Figure 5. Neuraminidases increase pneumococcal interaction with human lung cells. **A:** A549 cells infected with *S. pneumoniae* D39 and corresponding isogenic neuraminidase gene deletion mutants (left panel), or D39 wild-type in presence of α -neuraminidase mAbs (2 μ M each) or 4 μ M control IgG1 (middle panel). A549 cells treated with recombinant neuraminidases (1 nM each) or control protein (3 nM) and infected with D39 Δ nanA Δ nanB, or D39 wild-type and control protein (right panel). **B:** A549 cells infected with EF3030 or Sp#5 strains in the presence of α -neuraminidase mAbs (2 μ M each) or control IgG1 (4 μ M). **C:** Primary human lung tissues infected with *S. pneumoniae* EF3030 or Sp#5 in the presence of α -Ply (2 μ M) + α -neuraminidase mAbs (2 μ M each) or α -Ply (2 μ M) + control IgG1 (4 μ M). CFUs recovered from infected cells were calculated relative to those obtained with comparator strains or treatments indicated on the y-axes. Results from two independent experiments with at least biological triplicates (indicated by circles and squares) are depicted as mean with standard deviation.

Figure 6. Neuraminidases of different *S. pneumoniae* isolates desialylate murine lungs *in vivo*. Mice infected intranasally with *S. pneumoniae* D39, or D39 Δ nanA Δ nanB (**A**), or *S. pneumoniae* D39 + both α -neuraminidase mAbs (**B**), or *S. pneumoniae* EF3030 or Sp#5 in the presence of individual α -neuraminidase mAbs (**C**) for the indicated times. Arrows indicate desialylation as visualized by Lectin-FITC staining (green). Nuclei were stained with Hoechst dye (blue). Representative lung images of each treatment group are shown. Numbers indicate the observed frequencies of desialylation. Scale bar = 500 μ m. **C:** Semi-quantitative scoring of conducting and respiratory zone desialylation by EF3030 and Sp#5 in presence of α -neuraminidase mAbs.

1012 **Table 1. Recombinant proteins used in this study.** Neuraminidase protein characteristics are
 1013 outlined in the table below.

protein name	strain	GenBank	domains removed	characteristics
NanA-LD-SD	D39	ABJ55437.1	signal peptide, C-terminus	“wild-type” sequence
NanA-LDvar-SD	70585	ACO17645.1	signal peptide, C-terminus	most amino acid variations in lectin domain (LD)
NanA-LD-SDvar	INV200	CBW35147.1	signal peptide, C-terminus	most amino acid variations in sialidase domain (SD)
NanB	D39	ABJ55283.1	signal peptide	
NanC	TIGR4	AAK75424.1	signal peptide	

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Figure 1

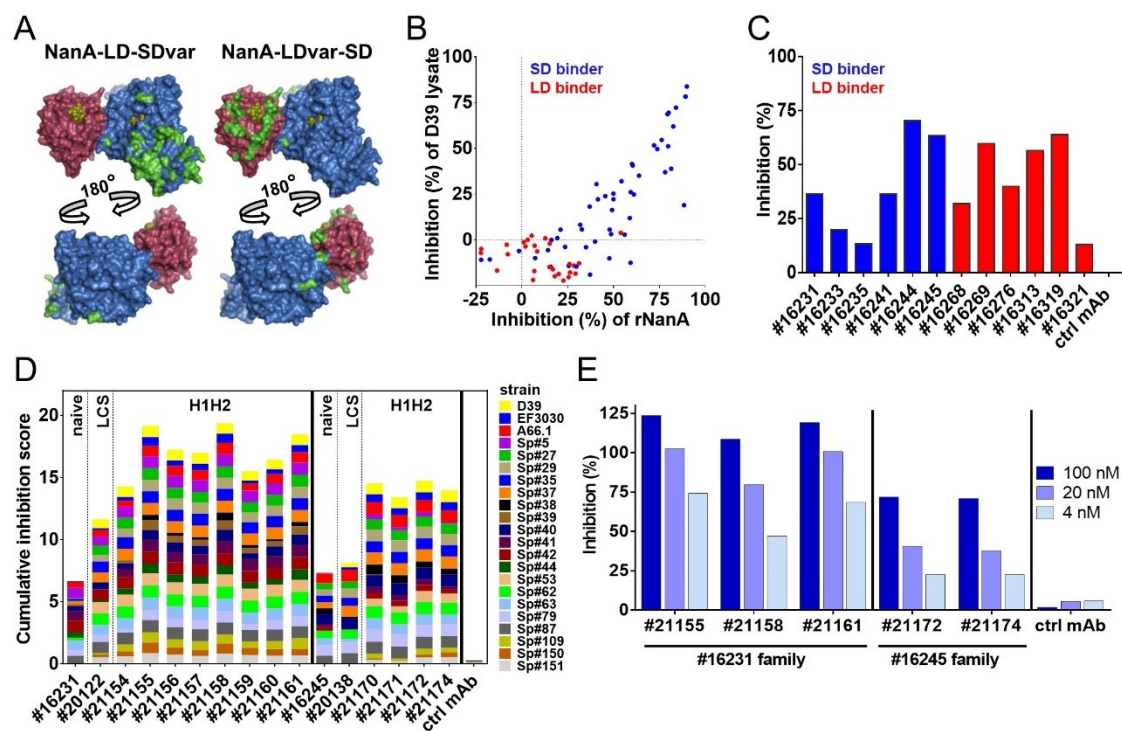


Figure 2

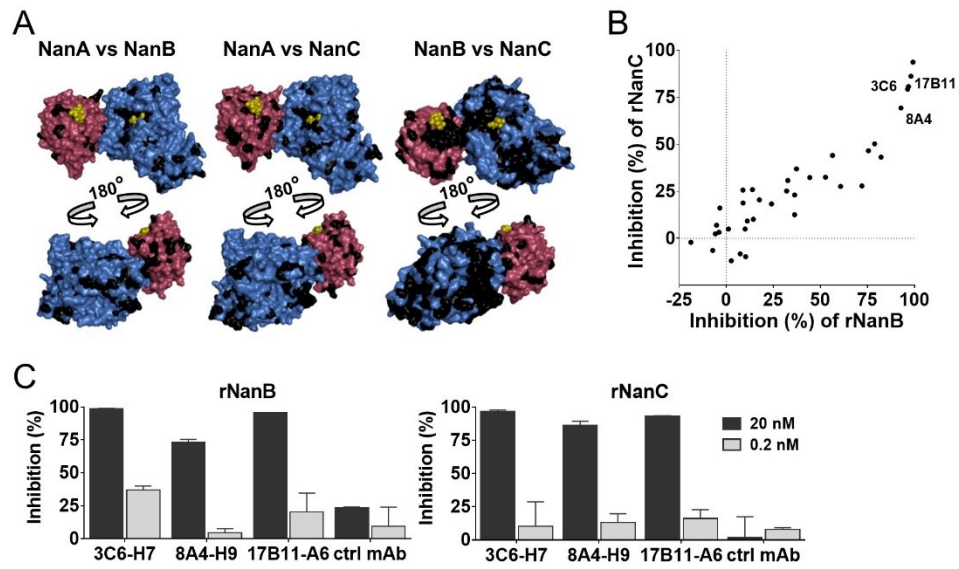


Figure 3

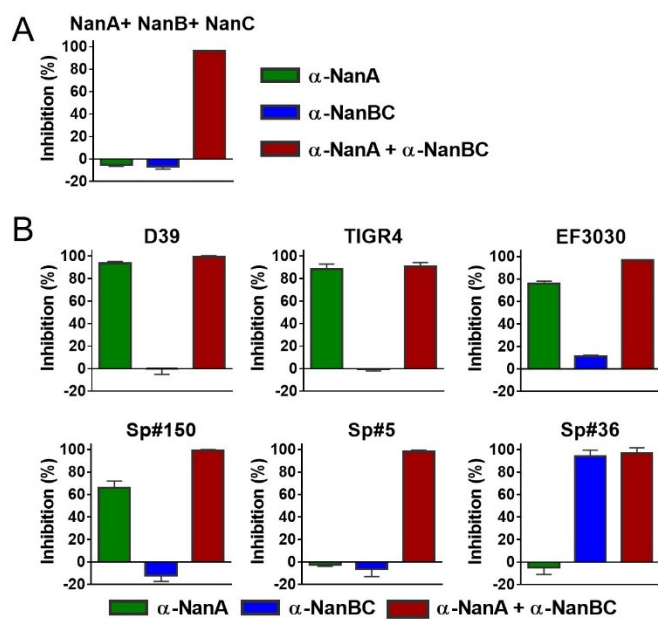


Figure 4

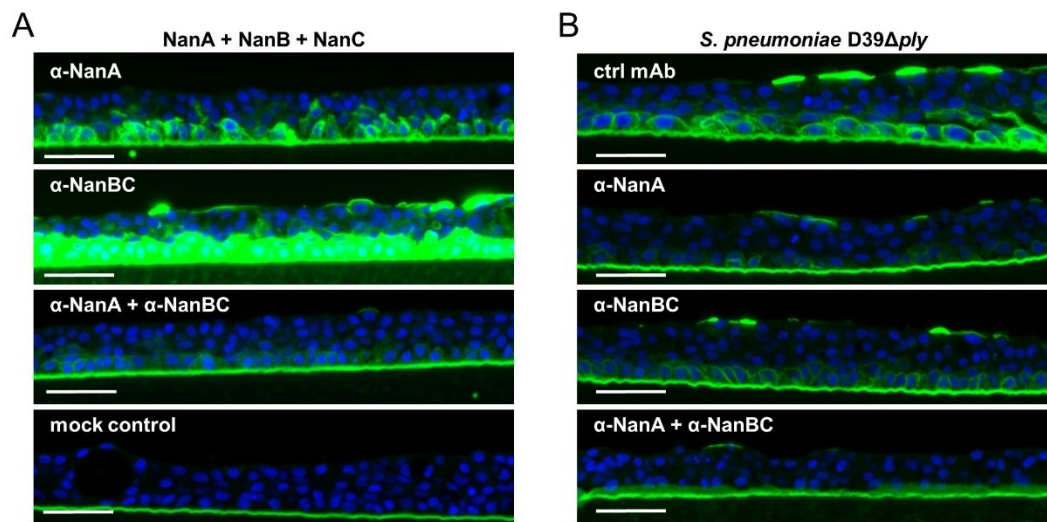


Figure 5

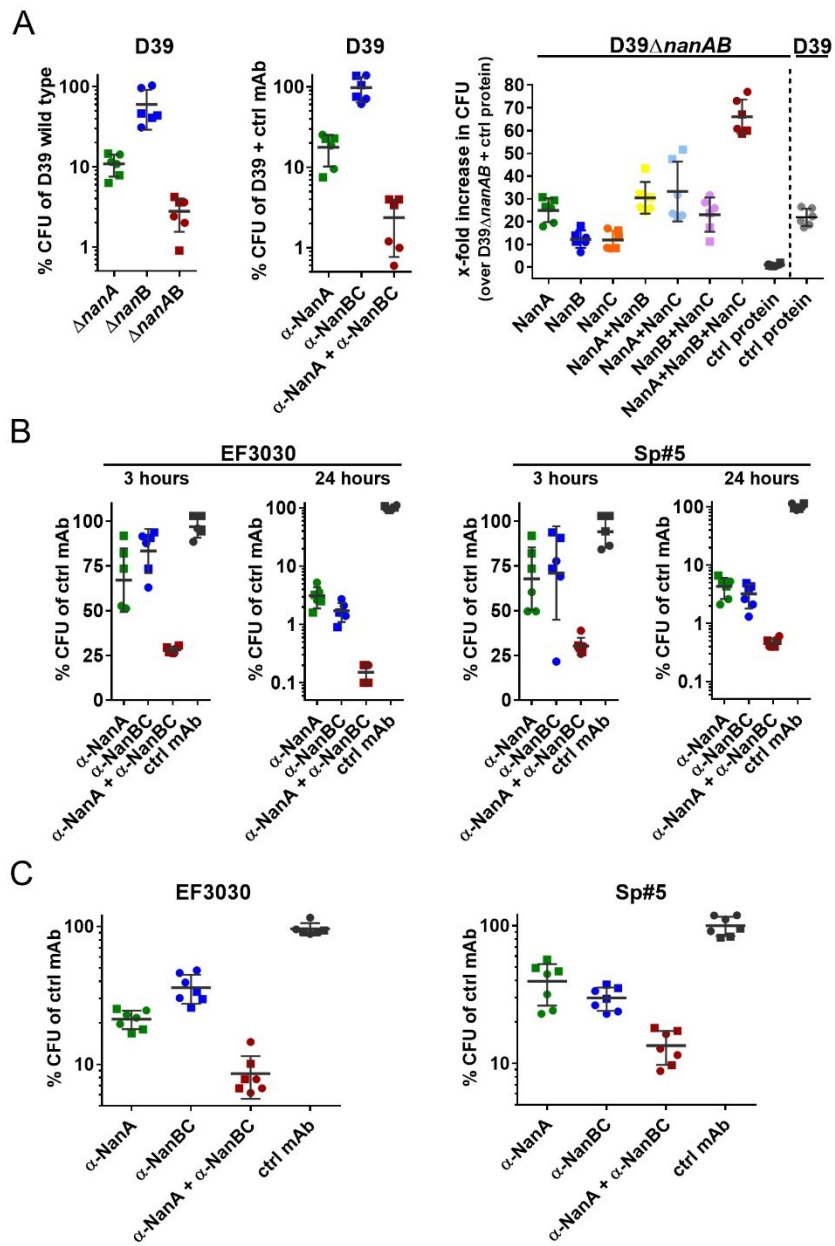


Figure 6

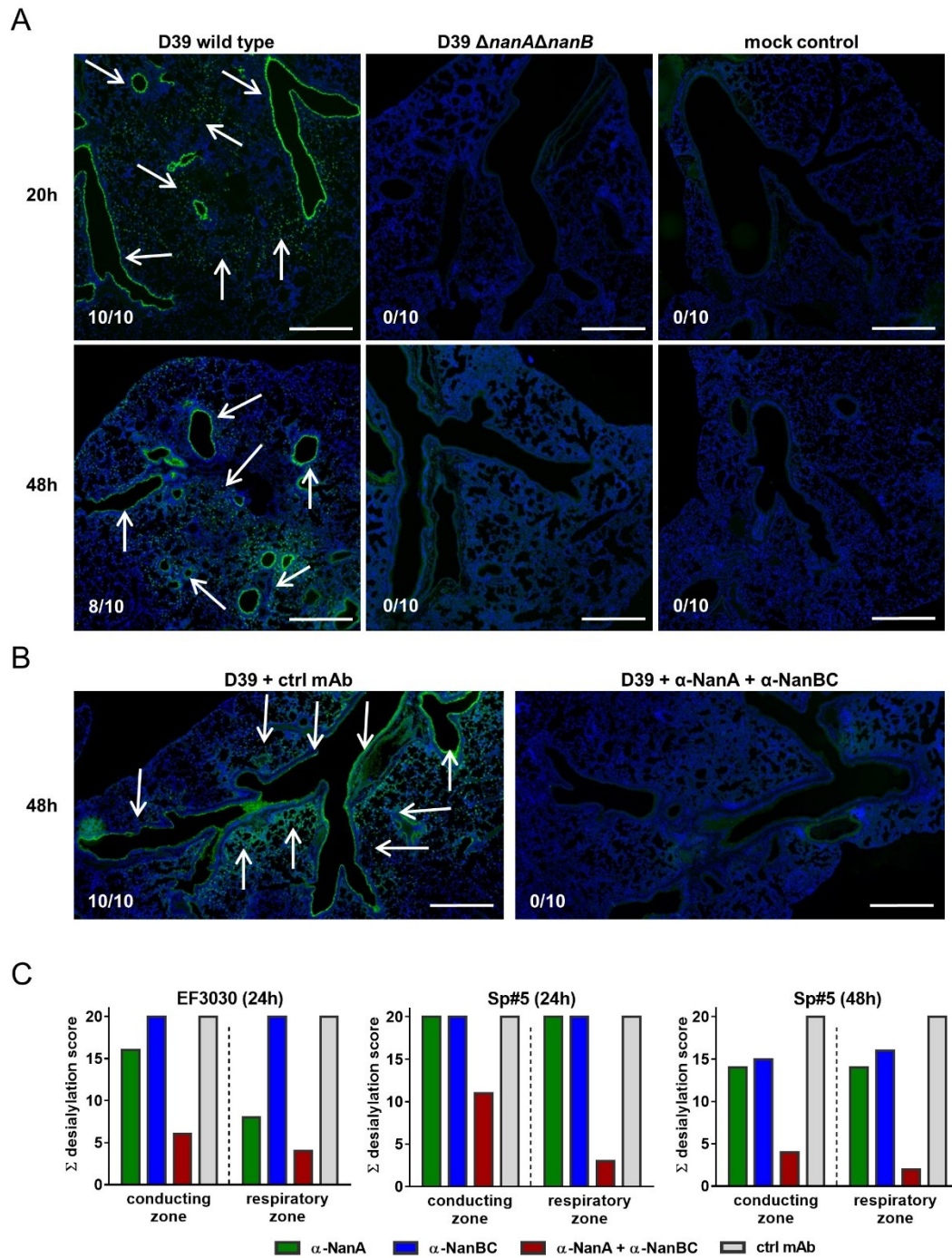


Figure S1

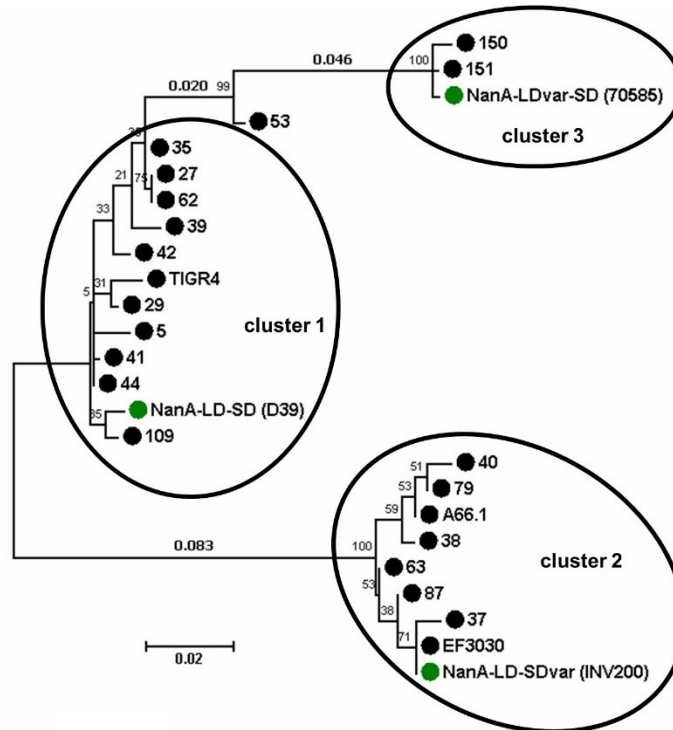


Figure S1. NanA sequence diversity. Evolutionary tree (generated with MEGA7.0.26, Maximum likelihood method, WAG model, Bootstrap $n=500$ replicates) of NanA (without the C-terminal-anchor) from *S. pneumoniae* strains (Sp#) used in this study (black), and NanA sequence variants (green). Numbers on branches indicate evolutionary distance (substitutions per site), Bootstrapping values are shown next to branch nodes.

Figure S2

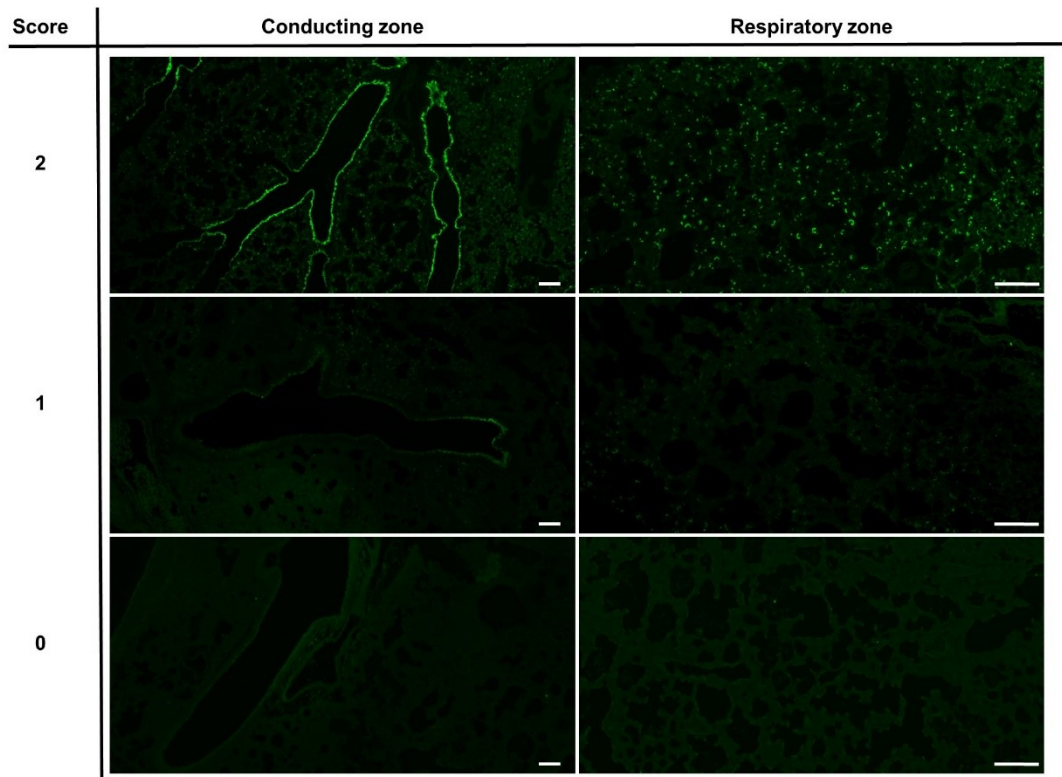


Figure S2. Example images for semi-quantitative scoring of lung desialylation. Mice (n=10) infected intranasally with *S. pneumoniae* in presence of α -NanA and α -NanBC or control mAb. Host sugar residues exposed by pneumococcal neuraminidases are stained with FITC-labeled peanut lectin (green). Scale bar= 100 μ m. Desialylation scores were assigned as follows: 2 (desialylation), 1 (reduced desialylation), or 0 (no desialylation). Conducting zones (comprising main bronchi and terminal bronchioles) and respiratory zones (comprising respiratory bronchioles, alveolar areas and interstitial cells) were scored independently. Cumulative scores of ten mice per treatment (maximum score= 20, minimum score= 0) are summarized in Fig. 6C.

Figure S3

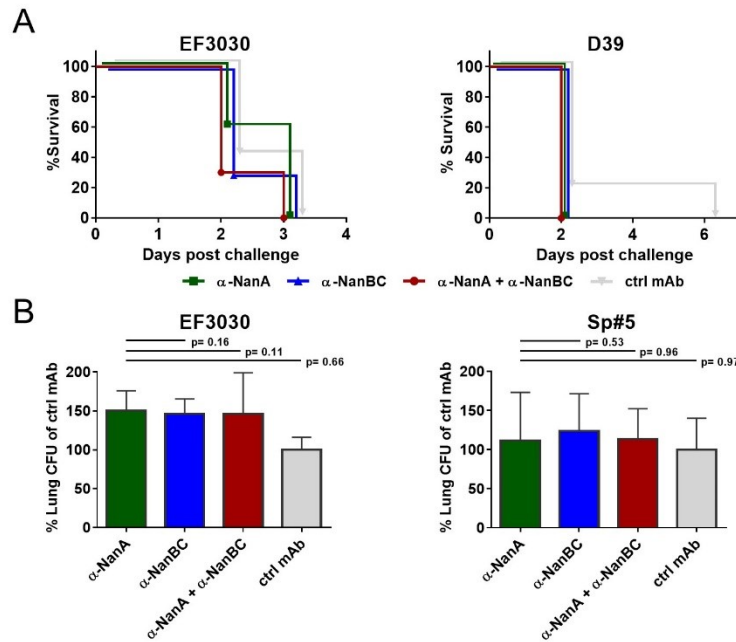


Figure S3. Targeting neuraminidases during murine acute pneumonia does not ameliorate disease.

A: Survival of mice infected with *S. pneumoniae* EF3030 or D39 strains.

B: Bacterial loads in lungs of mice infected with EF3030 or Sp#5 strains for 24 hours. Results are summarized as mean values relative to the control mAb treated groups $\pm$ SEM. Statistical differences were evaluated via Mann-Whitney test, p values are indicated on the graphs.

Table S1

Human clinical <i>S. pneumoniae</i> isolates						
strain name	sample origin	serotype	PspA clade	ply allele	nan genotype	NanA cluster (Figure S1)
Sp#5	nose	23F	3	1	ABC	1
Sp#27	nose	35	1	2	AB	1
Sp#29	trachea	6A	not determined	2	ABC	1
Sp#35	trachea	11A	not determined	2	ABC	1
Sp#36	sputum	15A	5	unclassified	ABC	mutant
Sp#37	bronchus	15B	not determined	2	AB	2
Sp#38	BAL	3	1	1	AB	2
Sp#39	nose	7C	5	2	AB	1
Sp#40	sputum	18C	5	2	AB	2
Sp#41	mini BAL	1	1	5	AB	1
Sp#42	sputum	4	4	unclassified	AB	1
Sp#44	nose	42	1	2	AB	1
Sp#53	trachea	19F	4	15	AB	1/3
Sp#62	nose	33F	1	2	ABC	1
Sp#63	bronchus	10A	1	1	AB	2
Sp#79	sputum	28F	1	unclassified	AB	2
Sp#87	bronchus	35B	4	1	AB	2
Sp#109	bronchus	14	1	unclassified	ABC	1
Sp#150	nose	19A	4	1	ABC	3
Sp#151	trachea	6B	6	1	A	3
<i>S. pneumoniae</i> prototype strains						
strain name	sample origin	serotype	PspA clade	ply allele	nan genotype	NanA cluster (Figure S1)
D39	not recorded	2	2	1	AB	1
A66.1	not recorded	3	2	1	AB	2
EF3030	otitis media	19F	1	2	AB	2
TIGR4	blood	4	3	2	ABC	1
<i>S. pneumoniae</i> D39 gene deletion mutant strains						
strain name	serotype	genotype	PspA clade	ply allele	nan genotype	
D39Δply	2	D39 Δply::erm ^R	2	1	AB	
D39ΔnanA	2	D39 ΔnanA::erm ^R	2	1	B	
D39ΔnanB	2	D39 ΔnanB::kan ^R	2	1	A	
D39ΔnanAB	2	D39 ΔnanA::erm ^R ΔnanB::kan ^R	2	1	-	

Table S1. *Streptococcus pneumoniae* strains used in this study.

Table S2

Gene	Primer (5' - 3')		Purpose of use
<i>nanA</i>	<i>nanA_KO1</i>	TTGATTTATTTCTTCACTTATTTGGGATTG	mutation
	<i>nanA_KO2</i>	ctgataaatatgaacatgatgagtgatcgtAGAAACCGCTCCTACCGATAGTT	gene deletion
	<i>nanA_KO3</i>	gacccggctaggctggcggggtgcGAGAAGAATTCTAAACATTTGATTTTGAAAAAT	gene deletion
	<i>nanA_KO4</i>	TTTCATTCTCTAAATTAGTTTTGTACCGT	gene deletion
	<i>nanA_erm</i> insertion_CTRL_1	GATTCTGATTGTGAGCTTGATTATATTTCC	deletion control
	<i>nanA_erm</i> insertion_CTRL_2	AGTCTTTTTCTAATTGGAATTTAGTTTCA	deletion control
	<i>erm_1</i>	ACGATCACTCATCATGTTTCATATTATCAG	gene deletion/ deletion control
	<i>erm_2</i>	GCAACCCCGCCAGCCTAGCCGGGTC	gene deletion/ deletion control
<i>nanB</i>	<i>nanB_KO1</i>	GCTGTATGACAATATAGAACGTCTCCGATA	gene deletion
	<i>nanB_KO2</i>	tacctagatttagatgtctaaaaagcAAACATGGTTAAATACGGTGTTGTTGGA	gene deletion
	<i>nanB_KO3</i>	tataacaagacgaactccaattcactgtTTTCCTTCTTTCTGCTACTTCACAGATC	gene deletion
	<i>nanB_KO4</i>	TTGTTAGCAATTCCTATTCAATTGCC	gene deletion
	<i>nanB_kan</i> insertion_CTRL_1	GCGACAATCTTGATAAGAAAG	deletion control
	<i>NanB_kan</i> insertion_CTRL_2	TAGAATTGATGGTGCAAATAAATT	deletion control
	<i>kan_1</i>	AACAGTGAATTGGAGTTCGTCTTGTATA	gene deletion/ deletion control
	<i>kan_2</i>	GCTTTTGTAGACATCTAAATCTAGGTA	gene deletion/ deletion control
<i>ply</i>	<i>ply_KO1</i>	ACTTTGGAAATCTGAGTCAATCAGAAGACT	gene deletion
	<i>ply_KO2</i>	ctgataaatatgaacatgatgagtgatcgtCTTCTACCTCCTAATAAGTTCCTGGATTGA	gene deletion
	<i>ply_KO3</i>	gacccggctaggctggcggggtgcGAGAATGCTTGCACAAAAAGAGGCGATGATCT	gene deletion
	<i>ply_KO4</i>	AGAAGAGTTTCTAAGCAAACCTCTAATACTCA	gene deletion
	<i>ply_mutag_CTRL_1</i>	GGATTATATTGTCAAGGTTATTGGCGACAAGCAT	deletion control
	<i>ply_mutag_CTRL_2</i>	GTCTACTCCAGACATATCATAGTTCAAGTAAATA	deletion control

Table S2. Oligonucleotide primers used for gene deletion mutant generation. Lowercase DNA sequences denote primer sequences overlapping with the respective 5' and 3' sequences of the corresponding antibiotic resistance cassette.

Table S3

Gene	Primer (5' - 3')		Purpose of use	Source
<i>nanA</i>	Fw	ATAGACGTGCGCAAAATACAGAATCA	Screening/ sequencing	Pettigrew et al. (2006)
	Rev	GTCGAACTCCAAGCCAATAACTCCT	screening	Pettigrew et al. (2006)
<i>nanB</i>	Fw	ACTACGAGGTGTTAATCGTAAGG	screening	Pettigrew et al. (2006)
	Rev	CCAATACCCGCAGGCATAACATC	screening	Pettigrew et al. (2006)
<i>nanC</i>	Fw	TGGGGTAAGTACAAACAAGAGG	screening	Pettigrew et al. (2006)
	Rev	CTAATGGTACTGGCGAAAATCA	screening	Pettigrew et al. (2006)
<i>nanA</i>	31R	TAACAGTACACCTTGACTGC	sequencing	Smith et al. (2013)
	31S	GTGTTTGATAAGGATTGAGC	sequencing	Smith et al. (2013)
	A_seq_fw3	TTGCGCACGTCTATTGTT	sequencing	this study
	A_seq_rev3	TGGGGTGATATCGGTATGG	sequencing	this study
	A_seq_rev4	AATGCTTCAAATGGTCAGAG	sequencing	this study
	Sp_seq1	GCATCGTATGGATAGCAG	sequencing	this study
	Sp_seq2	CTTGAACCAACACCATATC	sequencing	this study
<i>nanB</i>	B_seq_1	TCATCGCTAGAAACCAAC	sequencing	this study
	B_seq_2	TCTTACCTGTAGTGGTTCT	sequencing	this study
	B_seq_3	GGCATAACATCAGCTAGTAAT	sequencing	this study
	B_seq_4	TGTATTTTCAACTGCCTG	sequencing	this study
	B_seq_5	TCGGAAGTTTCGTATATTG	sequencing	this study
	B_seq_6	AAACTAAAGAAGAATGGAGATA	sequencing	this study
	B_seq_7	GTGTTCTCTGAAACAGTTGA	sequencing	this study
	B_seq_8	GGGAGATATGATGGCAG	sequencing	this study
<i>nanC</i>	C_seq_1	ACAAACATCTGATCCCAAC	sequencing	this study
	C_seq_2	ATGCTTGAATTACTCCTGG	sequencing	this study
	C_seq_3	AAAAC TACTCCAGCAGG	sequencing	this study
	C_seq_4	TTTGACTGAATCTGCTACTAC	sequencing	this study
	C_seq_5	CCCGGTCAACAAAATAGT	sequencing	this study
	C_seq_6	TAACATTGGCATTTCGATG	sequencing	this study
	C_seq_7	GGAATCATTGAAAGCTCA	sequencing	this study
	C_seq_8	ACGTGTACCTTTTAGCGA	sequencing	this study

Table S3. Oligonucleotide primers used for *nanABC* gene screening and sequencing.

Table S4

mAb	antigen	KD (M)	kon (M ⁻¹ s ⁻¹)	koff (s ⁻¹)
#21155	NanA-LD-SD	1.34E-09	1.49E+05	2.00E-04
	NanA-LDvar-SD	3.96E-09	1.31E+05	5.19E-04
	NanA LD-SDvar	2.42E-09	1.29E+05	3.11E-04
3C6-H7	NanB	5.86E-11	4.81E+05	2.82E-05
	NanC	9.58E-10	1.95E+05	1.86E-04

Table S4. Equilibrium and kinetic binding constants of α -neuraminidase mAbs to their target antigens.

Manuscript II

Age-related changes in the levels and kinetics of the pulmonary cytokine and chemokine responses to *Streptococcus pneumoniae* in mouse pneumonia models

“Life would be infinitely happier if we could only be born at the age of eighty and gradually approach eighteen.”

Mark Twain

Background and summary:

Due to improved living conditions and medical progress, human life expectancy has dramatically increased around the world. Aging is associated with immune senescence and declining defense functions increase the likelihood of severe infections, including *S. pneumoniae* infections, such as pneumonia. A hallmark of pneumococcal pneumonia is the excessive inflammatory response that can greatly harm the host. The elderly population is particularly vulnerable to pneumococcal pneumonia which has been linked to inappropriate inflammatory reactions⁴⁸. Although studies using aged mouse models of pneumococcal pneumonia have indicated that increased disease severity in this age group is accompanied by altered inflammatory responses, conflicting results have been reported with regards to their magnitude^{119–122}.

Thus, this study aimed at addressing how the kinetics of inflammatory responses, assessed by the production of pulmonary cytokines and chemokines, are affected by age during murine *S. pneumoniae* pneumonia. Aged compared to young mice had increased levels of inflammatory mediators in the absence of infection. In response to intranasal *S. pneumoniae* infection, however, aged mice showed delayed pulmonary immune responses, which became excessive over time. This was paralleled by bacterial growth that could not be contained, bacterial invasion and host mortality. On the contrary, young mice could control pneumococcal replication at the early stages of infection more efficiently, which was accompanied by sharp inflammatory responses, but importantly also subsequent resolution. Interestingly, several chemokines related to IFN- γ (MIG, IP-10, MCP-1, MDC, TARC) showed elevated levels in young but not aged mice early during infection. Since improved control of pneumococcal replication in young mice was already visible at this time point, elevated IFN- γ -related responses seem to be beneficial during early host responses.

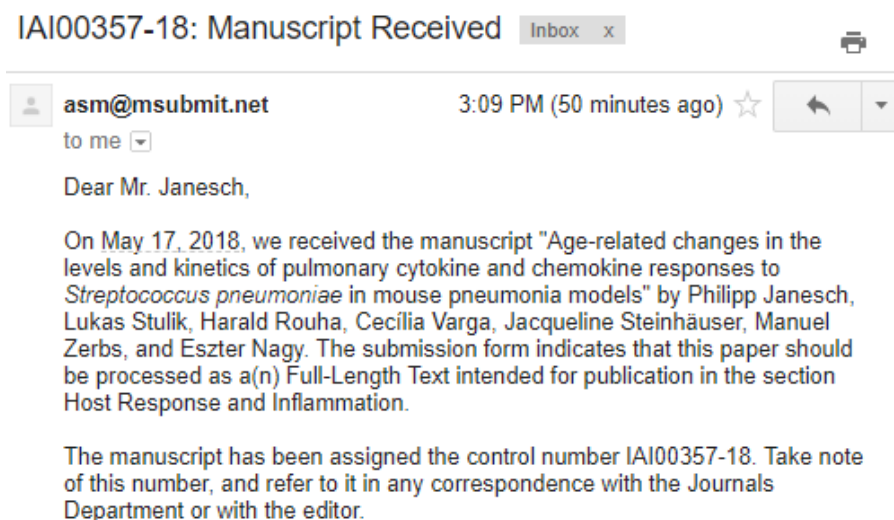
An important learning from these findings is that studies evaluating inflammatory reactions during bacterial infection of different age groups should not draw conclusions based on a single time point.

Personal contributions:

Performing the experiments shown in Figure 4 & 5, Table S1; data analysis, manuscript and figure preparation

Confirmation of manuscript submission:

This manuscript entitled “Age-related changes in the levels and kinetics of the pulmonary cytokine and chemokine responses to *Streptococcus pneumoniae* in mouse pneumonia models” was submitted to Infection & Immunity (IAI) on May 17th, 2018. It is currently under review (status May 18th, 2018). The confirmation received from the journal by e-mail (shortened for purpose of this dissertation) including the manuscript number is shown below:



Age-related changes in the levels and kinetics of pulmonary cytokine and chemokine responses to *Streptococcus pneumoniae* in mouse pneumonia models

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Running title: Altered anti-pneumococcal host responses in aged mice

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ABSTRACT

Streptococcus pneumoniae is a major human pathogen at the extremes of age. The elderly are particularly vulnerable to *S. pneumoniae*, the most common causative agent of bacterial pneumonia in this population. Despite the availability of vaccines and antibiotics, mortality rates associated with pneumococcal pneumonia in this age group remain high. In light of globally increasing life-expectancy, a better understanding of the patho-mechanisms of elderly pneumococcal pneumonia, including alterations in innate immune responses, is needed to develop improved therapies.

In this study we aimed at investigating how increased susceptibility to pneumococcal infection relates to inflammation kinetics in the aged mouse pneumonia model by determining pulmonary cytokine and chemokine levels and comparing these parameters to those measured in young adult mice.

Firstly, we detected overall higher pulmonary cytokine and chemokine levels in aged mice. However, upon induction of pneumococcal pneumonia in aged mice, delayed production of certain analytes, such as IFN- γ , MIG (CXCL9), IP-10 (CXCL10), MCP-1 (CCL2), TARC (CCL17) and MDC (CCL22) became apparent. In addition, aged mice were unable to control excess inflammatory responses: while young mice showed peak inflammatory responses at 24 hours and subsequent resolution by 48 hours post intranasal challenge, in aged mice increasing cytokine and chemokine levels were measured. These findings highlight the importance of considering multiple time points when delineating inflammatory responses to *S. pneumoniae* in an age-related context. Finally, correlation between pulmonary bacterial burden and cytokine or chemokine levels in young mice suggested that appropriately controlled inflammatory responses support the host to fight pneumococcal infection.

INTRODUCTION

Lower respiratory tract infections were reported to claim 2.74 million lives in 2015 and are the fifth leading cause of death world-wide (1) with community-acquired pneumonia (CAP) being the most common cause. Children under the age of five and elderly people (> 65 years of age) are disproportionally severely affected (2). Since 1980, the number of people above 60 years of age has increased globally from 382 to 962 million and is expected to double again by 2050 exceeding 2 billion (3). Given this prolonged life-expectancy, numbers of CAP cases in the elderly are expected to increase proportionally (4). Aging is not only associated with increased risk of CAP, but also high mortality rates and poor patient outcomes (5).

Numerous respiratory pathogens can cause CAP, but *Streptococcus pneumoniae* (the pneumococcus) is by far the most common causative agent isolated from adult CAP patients (5–7). Since the introduction of pneumococcal vaccines, rates of childhood pneumococcal diseases have been reduced significantly and also have had a positive impact on invasive pneumococcal disease rates (such as bacteraemia, septicaemia) in the elderly with great variation between countries (8–10). Development of non-bacteremic pneumococcal pneumonia in this age group has remained largely unaffected (11, 12). Case fatality rates of pneumococcal pneumonia range from 10 to 15 % in patients above 65 years of age, thereupon increasing by another 10 % with every decade of life (13). The enhanced susceptibility to pneumococcal pneumonia is mainly attributed to age-related co-morbidities, impaired mechanical clearance of the bacterium from the respiratory tract (14), viral co-infections such as influenza (15) and importantly, immune senescence (16).

Many factors contribute to impaired age-related immune responses, which predispose the elderly to disease. These include both innate and adaptive immune dysfunction, such as impaired Toll-like receptor (TLR) signaling (17), reduced neutrophil and lymphocyte

chemotaxis, phagocytosis and natural killer (NK) cell activity (18), alterations in B-cell responses (19) and immune cell-type polarization (20). In the context of *S. pneumoniae* infection, macrophage (21, 22) or monocyte dysfunction (23), as well as changes in the natural IgM repertoire (24) and reduced opsonophagocytic activity of neutrophils (25) were reported. Moreover, chronic low-grade age-related inflammation increases susceptibility to pneumococcal pneumonia by upregulating epithelial surface receptor expression (such as laminin receptor, platelet-activating factor receptor, or keratin-10) that the pneumococcus utilizes for attachment (26).

Host inflammatory networks governed by cellular crosstalk *via* cytokines and chemokines are essential to contain pneumococcal infections. Not only the magnitude of these responses, but also the subsequent resolution, are important features to ensure recovery and tissue integrity (27). It has been reported that aged mice (similar to elderly humans) compared to young mice are more susceptible to pneumococcal pneumonia (21, 23, 26, 28, 29).

The extent and kinetics of inflammatory responses to *S. pneumoniae* can differ substantially locally and systemically (30, 31) and the effects of aging on cytokine and chemokine dynamics during severe pneumococcal pneumonia have been largely unexplored. We therefore compared the pulmonary levels of inflammatory mediators during *S. pneumoniae* pneumonia in young and aged mice at different time points following the induction of pneumonia.

MATERIALS & METHODS

Preparation of *S. pneumoniae* inocula. *S. pneumoniae* strain D39 (serotype 2) was kindly provided by Dr. David Briles (University of Alabama at Birmingham, U.S.) and used in all experiments. Bacterial stocks were generated by growing *S. pneumoniae* D39 in Todd-Hewitt Broth supplemented with 0.5 % yeast extract (THY; Sigma) to OD_{620nm} 0.4 (37 °C, 5 % CO₂) and stored in 25 % glycerol at -80 °C. For the preparation of challenge inocula, stocks were thawed on ice, bacterial pellets were washed twice in sterile PBS and adjusted to the expected challenge inoculum in PBS. The actual challenge inoculum was confirmed in each experiment by serial plating on Columbia agar supplemented with 5 % sheep blood (COS, Biomerieux) and enumeration after overnight incubation (37 °C, 5 % CO₂).

Murine *S. pneumoniae* pneumonia models. Animal experiments were performed according to Austrian Law (BGBl. I Nr. 114/2012, approved by MA58, Vienna, Austria) and IACUC approval, with female, 6 to 8 weeks or 18 to 21 months old C57BL/6JRj mice (Janvier, France). These life-phases correspond to human age equivalents of approximately 20-30, or 55-70 years, respectively (32).

Mice were anesthetized with ketamine/xylazine followed by drop-wise intranasal application of the bacterial challenge inoculum (2×10^7 CFU/ 40 μ L of *S. pneumoniae* D39). Survival was monitored for up to ten days post-infection. For microbiological and immunological readouts, animals were weighed, challenged (2×10^7 CFU/ 40 μ L of *S. pneumoniae* D39) and sacrificed by overdosing of isoflurane at 20 or 48 hours post-infection. Mice were weighed, blood and organs were harvested. Organs were washed in PBS and weighed prior to homogenization (Precellys 24 homogenizer) in Precellys tubes filled with 5 mL PBS and 1.44 mm zirconium oxide bulk beads. All samples were processed on ice.

Bacterial CFU was determined after serial plating on COS plates and incubation overnight (37 °C, 5 % CO₂). Proteinase inhibitor cocktail (Sigma) was added to the samples, which were centrifuged (13.000 rpm, 15 min, 4 °C) to harvest supernatants. All samples were aliquoted and subsequently stored at -80 °C until further analysis.

Lung cytokine and chemokine measurements. Levels of pulmonary inflammatory markers were measured using the LEGENDplex™ Mouse Inflammation Panel and Mouse Proinflammatory Chemokine Panel (BioLegend) multiplex bead arrays according to the manufacturer's instructions. Samples were measured on a CytoFLEX flow cytometer (Beckman Coulter) and results were analyzed using LEGENDPlex software 7.0. Analyte levels were normalized to lung weights. The analytes and detection limits were as follows: RANTES/ CCL5 (1.17 pg/mL), MIP-3α/ CCL20 (1.24 pg/mL), Eotaxin/ CCL11 (1.43 pg/mL), TARC/ CCL17 (1.34 pg/mL), KC/ CXCL1 (1.28 pg/mL), MCP-1/ CCL2 (1.38 pg/mL), MIG/ CXCL9 (1.88 pg/mL), IP-10/ CXCL10 (1.26 pg/mL), MIP-1α/ CCL3 (1.63 pg/mL), MIP-1β/ CCL4 (1.38 pg/mL), BLC/ CXCL13 (1.45 pg/mL), LIX/ CXCL5 (7.99 pg/mL), MDC/ CCL22 (1.27 pg/mL), IL-23 (6.71 pg/mL), IL-1α (1.19 pg/mL), IFN-γ (1.30 pg/mL), TNF-α (1.25 pg/mL), IL-12p70 (0.62 pg/mL), IL-1β (0.61 pg/mL), IL-10 (1.22 pg/mL), IL-6 (0.64 pg/mL), IL-27 (10.93 pg/mL), IL-17A (1.22 pg/mL), IFN-β (3.75 pg/mL), GM-CSF (6.62 pg/mL). Given that the mean lung weights were approximately 150-300 mg and that lungs were homogenized in 5 mL PBS, the above values correspond to lower limits of detection between ~ 0.02 and 0.2 pg/ mg lung tissue.

Statistical analysis. All analyses were performed using Prism® 6.07 software (GraphPad). Differences in mouse survival were evaluated using Log-rank (Mantel Cox) test. Differences in body weight changes were evaluated by Wilcoxon matched-pairs signed rank test, while

lung weights, edema scores, bacterial burden or inflammatory analyte levels were analyzed using Mann-Whitney U-test (two-tailed p-values). Changes in levels of inflammatory analytes were calculated relative to the corresponding control group (mock challenge with PBS). When an analyte was not detectable (values below the detection limit), it was equated with the lower limit of detection to enable calculation of the (minimal) fold-changes. The median ratios, calculated as $\log_2$ (infected / mock values) as previously published (33) were visualized using Permut-Matrix software 1.9.4 (34). Correlation analyses are shown as Spearman's coefficient (r) with two-tailed p-values.

RESULTS

Higher mortality of aged vs. young mice upon intranasal challenge with *S. pneumoniae*.

To confirm the notion that aged mice are more susceptible to pneumococcal pneumonia than their younger counterparts, we compared lethality rates in aged and young mice upon intranasal challenge with a high dose of *S. pneumoniae* D39 (2×10^7 CFU/animal). All aged mice succumbed to infection by day 4, whereas 50 % of the young animals were still alive at this time point. Additional deaths occurred until day 6 post-infection, but 40 % of animals survived until the end of monitoring (day 10) (Fig. 1). The difference in death rates was significant ($p < 0.0001$).

Control of *S. pneumoniae* growth is impaired in aged mice. To determine whether the observed age-related differences in lethality arose from a reduced control of pneumococcal growth, the bacterial organ load at 20 and 48 hours post intranasal infection with the *S. pneumoniae* D39 strain was evaluated.

At 20 hours post-infection, body weight changes were more pronounced in young compared to aged animals, which equalized between the age-groups by 48 hours (Fig. 2A). Lung weight changes of infected vs. control animals at 20 hours post-infection were comparable between young and aged mice, whereas a tendency towards higher lung weights of aged mice was observed at 48 hours, without reaching statistical significance (Fig. 2B). The same trend was observed for edema scores (Fig. 2C).

Although young mice contained bacterial growth in the lung more efficiently than aged animals (group medians differing by > 1 log) at the early stages of infection, bacterial numbers in the bloodstream and spleens were comparable (Fig. 3). At the later time point, pneumococcal numbers greatly increased in aged mice in the lung (by ~ 2 -log), as well as in blood and spleen (by > 4 -log). Bacterial outgrowth was significantly better contained in young animals, although they showed heterogeneity ranging from complete clearance to intermediate or high (comparable to those found in aged mice) bacterial loads. Young mice that showed the highest bacterial burden in the lung at 48 hours post-infection were the same animals that had the highest bacterial numbers in the blood and spleens.

These data suggest that local clearance of *S. pneumoniae* in the lungs of aged mice is impaired, which leads to earlier systemic spread.

Aged mice display delayed kinetics of pulmonary inflammatory responses and fail to resolve inflammation. To detect potential differences in inflammatory responses of aged versus young mice to pneumococcal infection, we determined the pulmonary levels of 25 cytokines and chemokines (same animals as for CFU enumeration).

The general notion that aging is associated with chronic inflammation, was confirmed by our data as approximately two thirds of the cytokines and chemokines detected in the lung tissue were significantly elevated in aged compared to young animals (Table S1).

Upon bacterial challenge, young mice reacted with a robust pulmonary inflammatory response at 20 hours post-infection, which greatly decreased by 48 hours. On the contrary, in aged mice the levels of 21 out of 25 cytokines and chemokines either increased over time (e.g. KC), remained unchanged (e.g. TNF- α), or in the case of IL-1 α were not reduced to the same levels as observed in young mice (Fig. 4A, B).

The B lymphocyte chemoattractant (BLC aka CXCL13) was the only inflammatory marker that increased in both age groups from 20 to 48 hours post-infection and was significantly elevated in aged mice at both time points (Fig. 4C).

Interestingly, the concentrations of most analytes were comparable between the age groups at 20 hours post-infection, however, six were significantly higher in young mice: IFN- γ , MIG (Monokine induced by gamma interferon; CXCL9), IP-10 (Interferon gamma induced-protein 10; CXCL10), MCP-1 (Monocyte chemoattractant protein-1; CCL2), TARC (Thymus and activation regulated chemokine; CCL17) and MDC (Macrophage-derived chemokine; CCL22) (Fig. 4D). By 48 hours post-infection, this trend was reversed, except for TARC and MDC, which were still higher in young compared to aged mice, although lower compared to the earlier time point.

IL-10, IL-17A, IL-27, or GM-CSF expression could not be detected in most young mice. In aged mice, expression of IL-10, IL-27 and to a lesser extent GM-CSF became evident (with minimal changes in IL-17A).

The heterogeneity in bacterial clearance from the lungs observed in young mice at 48 hours post-infection (Fig. 3) prompted us to correlate the levels of inflammatory markers with bacterial burden. The levels of the majority of cytokines and chemokines showed a strong positive correlation with the pulmonary bacterial load, apart from MDC and TARC, which negatively correlated (Fig. 5).

211 These data suggest that despite an elevated pro-inflammatory status in the absence of
212 infection, aged mice elicited delayed pulmonary inflammatory responses during severe
213 pneumococcal pneumonia which became excessive over time. Moreover, resolution of
214 inflammation 48 hours post-infection in young mice correlated with pneumococcal clearance.

215

216

DISCUSSION

It is well established that aging affects host defenses against bacterial infections. Respiratory pathogens and particularly *S. pneumoniae* are responsible for substantial morbidity and mortality in the elderly (> 65 years of age) (5–7). Age-associated chronic low-grade inflammation of the respiratory tract has been reported in humans (35) and mice (36). In the elderly, one of the classical symptoms of CAP, namely fever (induced by inflammatory mediators), is frequently absent at hospital admission (37, 38), which is associated with increased mortality rates (39).

Previous studies that addressed the differences in pulmonary inflammatory responses to *S. pneumoniae* in aged mouse models of pneumonia, made their observations based on a single time point after infection (21, 29, 40, 41). Depending on the model and the time point when pulmonary inflammation was assessed, the authors reached different conclusions. Reduced pro-inflammatory responses in aged mice were reported 4 to 6 hours post-challenge (21, 40), whereas increased pro-inflammatory responses were noted 24 to 48 hours post-challenge (29, 41). Our study connects and extends these findings by bringing these seemingly contradictory observations into a more dynamic kinetic context. In agreement with other studies (21, 29, 40) we found that aged mice were more susceptible to *S. pneumoniae* D39 pneumonia, had elevated pulmonary levels of several inflammatory markers in the absence of infection, contained bacterial replication in the lung less effectively at 20 hours post-infection and showed substantially increased bacterial loads with systemic dissemination at 48 hours post-infection.

Classically, the early host response to *S. pneumoniae* in the lung is initiated *via* the innate recognition by resident cells, which secrete cytokines and chemokines to recruit additional immune cells to the site of infection. Neutrophils are the first infiltrating cells that

are important for the early control of bacteria and dictate the subsequent recruitment of monocytes, macrophages and lymphocytes (30). Inflammatory responses early during pneumococcal lung infection are crucial for disease outcome and survival of the host (42). Once the pneumococcal burden reaches a critical level, the infection cannot be contained despite strong inflammatory reactions. In fact, uncontrolled inflammation can even harm the host and can result in reduced survival (30). This is underlined by an important study demonstrating that improved pneumococcal clearance from the lung in mice vaccinated with *S. pneumoniae* PspA was accompanied by a sharp increase in certain inflammatory mediators (such as TNF- α , IL-6, IFN- γ) and a much faster decline compared to controls (43). Likewise, IL-6 and TNF- α production early during pneumococcal infection followed by their rapid downregulation was associated with better survival in BALB/c mice (considered as the most resistant mouse strain to *S. pneumoniae* infections), whereas excess production and failure to tame the response was associated with enhanced susceptibility of CBA mice to pneumococci (44). In line with these findings, improved containment of *S. pneumoniae* in young mice in our study was paralleled by peak inflammatory responses at 20 hours post-infection and a subsequent decrease by 48 hours. However, in the aged counterparts increased levels of inflammatory markers were detected over time. Moreover, aged but not young mice showed strong IL-6 responses 48 hours post-infection together with the induction of IL-10 and IL-27 (both of which are classically viewed as anti-inflammatory), a combination that is a known predictor of poor clinical outcome in human CAP patients (45).

A growing body of evidence demonstrates that type-1 interferons (IFNs) are not only important for the early innate control of intracellular pathogens, but also for extracellular bacteria including *S. pneumoniae* (46). Sub-lytic concentrations of the pore-forming pneumococcal cytotoxin pneumolysin can induce cell damage, which leads to uptake of bacterial DNA and the initiation of type-I IFN signaling by cytosolic DNA sensors (47). Type-I

IFNs are directly involved in preventing pneumococcal transmigration from the lung to more distal sites, by reducing the expression of the platelet-activating factor receptor (PAFR) *via* inhibition of TNF- α and increasing expression of tight junction proteins (48). We observed similar changes in both TNF- α and IFN- β levels in aged and young mice at 20 hours post-infection. However, despite elevated IFN- β levels, aged mice did not show substantially decreased TNF- α levels at 48 hours, suggesting reduced responsiveness to this regulatory mechanism.

Moreover, it has been demonstrated in a *Klebsiella pneumoniae* lung infection model that type-I IFNs can promote type-II IFN (IFN- γ) production by NK cells, which is required to control bacterial elimination by phagocytes (49). Whether IFN- γ has such a direct role in *S. pneumoniae* pathogenesis is more controversial: C57BL/6 IFN- $\gamma^{-/-}$ mice were shown to be more susceptible to pneumococcal disease than wild-type animals (50), which was not observed in BALB/c IFN- $\gamma^{-/-}$ mice (51). IFN- γ production is classically considered as being dependent on IL-12 and mainly produced by professional antigen-presenting cells (APCs), NK cells and lymphocytes (52). However, it has recently been demonstrated that during pneumococcal pneumonia in C57BL/6 mice, neutrophils are the almost exclusive source of this cytokine in the lung (at 24 hours post-infection) and its secretion is independent of IL-12 (53, 54). Our data demonstrate that young compared to aged mice show an elevated IFN- γ response at 20 hours post-infection, which reversed at 48 hours. This may be explained by accelerated PMN recruitment, which would need to be addressed in further studies.

Similar to our results, pulmonary MIG was previously shown to be strongly upregulated in pneumococcal pneumonia models (55). Induction of MIG depends on IFN- γ and can be further amplified by TNF- α . Apart from its role in Type 1 helper T cell (Th1) attraction, both human and murine recombinant MIG possess direct anti-pneumococcal activity *in vitro*. However, MIG $^{-/-}$ mice were not more susceptible to pneumococcal infection than wild-type

animals, presumably due to functional redundancy of two other chemokines (I-TAC and IP-10) that bind the same receptor, CXCR3 (55). Moreover, deletion of CXCR3 improved host outcome in a serotype-dependent manner (56). Interestingly, in our experiments, pulmonary IFN- γ , MIG (dependent on IFN- γ) and IP-10 (which can be induced by IFN- γ but also independently) were significantly elevated in young mice at the earlier time point when reduced bacterial burden in the lung was observed. This indicates that CXCR3 chemokines can directly interact with *S. pneumoniae* early after infection and independently of their role in T-cell attraction.

CD4⁺ T-cell and MCP-1-dependent monocyte recruitment to the lung is important for the early control of *S. pneumoniae* infections (57, 58). This is in line with our findings demonstrating that the MCP-1 and Th2 attracting chemokines (TARC, MDC) were strongly elevated early during infection in young, but not in aged mice. Importantly, IFN- γ has been reported to be involved in the induction of all of these chemokines (59, 60).

Inflammatory markers and bacterial burden in the lungs of young mice at 48 hours post-infection were in general positively correlated. However, MDC and to a lesser extent TARC (only borderline significant) were the only mediators that remained elevated in mice that contained the pneumococcal infection, suggesting sustained T-cell involvement.

The only inflammatory analyte that was elevated in the aged compared to young mice at every time point tested (absence of infection, 20 and 48 hours post-infection) was BLC, which is mainly involved in B-cell function and trafficking (61). This chemokine is increased in lungs of patients with idiopathic pulmonary fibrosis (62). Pneumococcal pneumolysin was reported to trigger progression of this disease in mice (63). Whether elevated BLC levels contribute to age-related susceptibility to pneumococcal pneumonia, warrants further investigation.

Although several studies have reported delayed inflammatory responses during respiratory infections in aged mice (21, 40, 64–66), *Francisella tularensis* lung infections were better contained by aged mice at the early stages of infection, despite an overall delayed local inflammatory response (65, 66). Additionally, aged mice showed improved control of *Mycobacterium tuberculosis* at the initial stages of infection (67). Similar to *S. pneumoniae*, reduced clearance of influenza virus in aged mice was accompanied by delayed inflammatory responses (64). It is therefore important to note that age-related delayed immune responses can differentially influence the early control of respiratory infections in a pathogen-specific manner but are clearly detrimental during *S. pneumoniae* pneumonia.

The most important conclusion of this study is that the kinetics of pulmonary inflammatory responses to pneumococcal infection greatly differ in young and aged animals. Therefore, it is utterly important to perform such analyses at different time points to gain a dynamic and comprehensive instead of a static picture.

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Conflict of interest

The authors declare a potential conflict of interest as they are employees of Arsanis Biosciences GmbH (Vienna, Austria) and shareholders in Arsanis, Inc (USA), a biotechnology company developing anti-infective monoclonal antibody-based products.

Acknowledgements

We thank David Briles for providing the *S. pneumoniae* strain D39.

FIGURE LEGENDS

Figure 1. Aged compared to young mice are more susceptible to pneumococcal pneumonia. Survival of aged and young mice after intranasal infection with 2×10^7 CFU *S. pneumoniae* D39 was monitored for up to 10 days post challenge. Data are derived from three independent studies with 3-5 mice/group per experiment (up to 15/group in total). Differences in mouse survival were evaluated using Log-rank (Mantel Cox) test.

Figure 2. Body and lung weight changes of mice in response to *S. pneumoniae* infection. Mice (n=5/group in 3 independent studies) were intranasally infected with 2×10^7 CFU of *S. pneumoniae* D39. **A:** Body weights of mice measured before (0 hours) and 20 or 48 hours post-infection. **B:** Lung weights determined at 20 and 48 hours post-infection. **C:** Edema scores (lung-to-body weight ratios) at 20 and 48 hours post-infection. Changes relative to the age-matched control animals are indicated as percentages. Independent experiments are shown (circles, squares and rectangles), bars indicate group median values. Group differences were statistically evaluated using Wilcoxon matched-pairs signed rank test or Mann-Whitney U-test; p-values are indicated on the graphs.

Figure 3. Impaired clearance of pneumococci from the lung and systemic dissemination in aged mice. Bacterial burden in lung, blood and spleen of infected animals were determined at 20 and 48 hours post-infection by CFU plating. Data are derived from the same three

independent studies as shown in Fig. 2. (marked by circles, squares and rectangles). Lower limit of detection ranges are marked by dotted lines, bars indicate group median values. Group differences were statistically evaluated using Mann-Whitney U-test; p-values are indicated on the graphs.

Figure 4. Aged mice display delayed and prolonged inflammatory responses to pneumococcal lung infection. Levels of pulmonary cytokines and chemokines in aged and young mice were measured at 20 or 48 hours following intranasal infection with 2×10^7 CFU *S. pneumoniae* D39. **A:** Changes in analyte levels calculated relative to the control groups as $\log_2$ (infected value/ mock value). Dynamics in expression levels depicted as heat map with a color scale indicating the median fold changes. Black color indicates median values below the lower limit of detection. **B:** Representative examples for different patterns of changes in analytes in aged and young mice. **C:** Increasing BLC levels in both age groups over time. **D:** Delayed upregulation of IFN- γ and related chemokines in aged mice. Data from the same three independent studies (marked by circles, squares and rectangles) as in Fig. 2 & Fig. 3 are shown, bars indicate group median values. Group differences were statistically evaluated using Mann-Whitney U-test; p-values are indicated on the graphs.

Figure 5. Correlation between bacterial burden and cytokine/chemokine levels in the lungs of young mice infected with *S. pneumoniae*. **A:** Bacterial load in lungs, blood and spleens of young mice at 48 hours post-infection as shown in Fig. 3. A subgroup of young mice completely cleared *S. pneumoniae* from the lung (green), showed similar or reduced bacterial burden compared to levels at 20 hours post-infection (yellow), or failed to contain bacterial growth (red) similar to aged mice. **B:** Correlation of inflammatory cytokine/chemokine levels

389 with bacterial lung burden (colors as in panel A) with Spearman coefficients r (two tailed-p
390 values). Analytes not shown were not detectable or did not show correlation.
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Figure 1

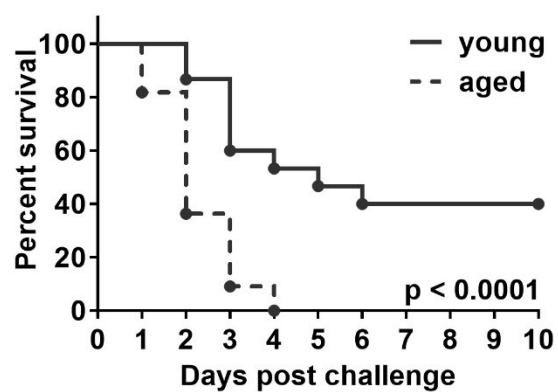


Figure 1. Aged compared to young mice are more susceptible to pneumococcal pneumonia. Survival of aged and young mice after intranasal infection with 2×10^7 CFU *S. pneumoniae* D39 was monitored for up to 10 days post challenge. Data are derived from three independent studies with 3-5 mice/group per experiment (up to 15/group in total). Differences in mouse survival were evaluated using Log-rank (Mantel Cox) test.

Figure 2

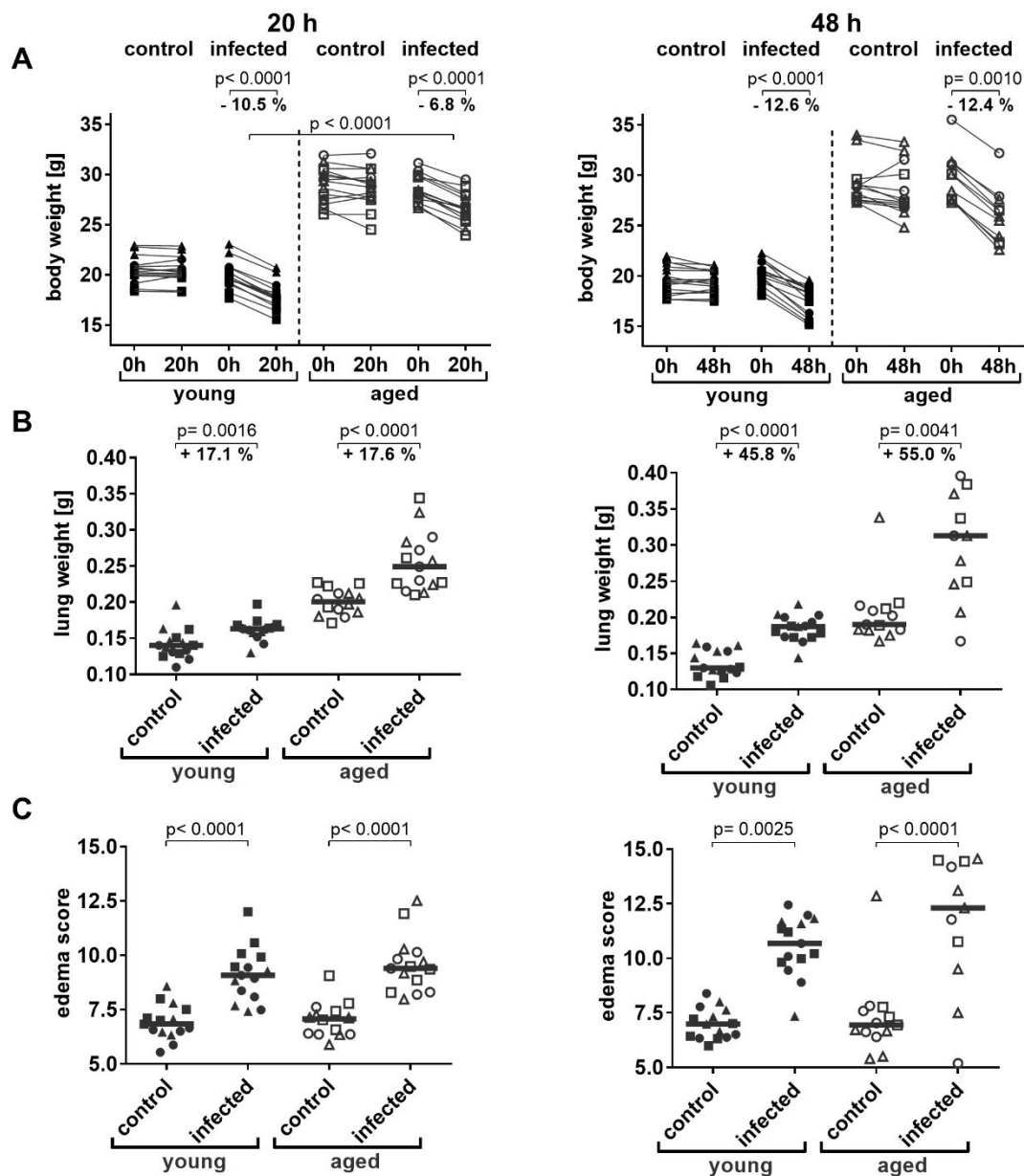


Figure 2. Body and lung weight changes of mice in response to *S. pneumoniae* infection. Mice ($n=5$ /group in 3 independent studies) were intranasally infected with 2×10^7 CFU of *S. pneumoniae* D39. **A:** Body weights of mice measured before (0 hours) and 20 or 48 hours post-infection. **B:** Lung weights determined at 20 and 48 hours post-infection. **C:** Edema scores (lung-to-body weight ratios) at 20 and 48 hours post-infection. Changes relative to the age-matched control animals are indicated as percentages. Independent experiments are shown (circles, squares and rectangles), bars indicate group median values. Group differences were statistically evaluated using Wilcoxon matched-pairs signed rank test or Mann-Whitney U-test; p-values are indicated on the graphs.

Figure 3

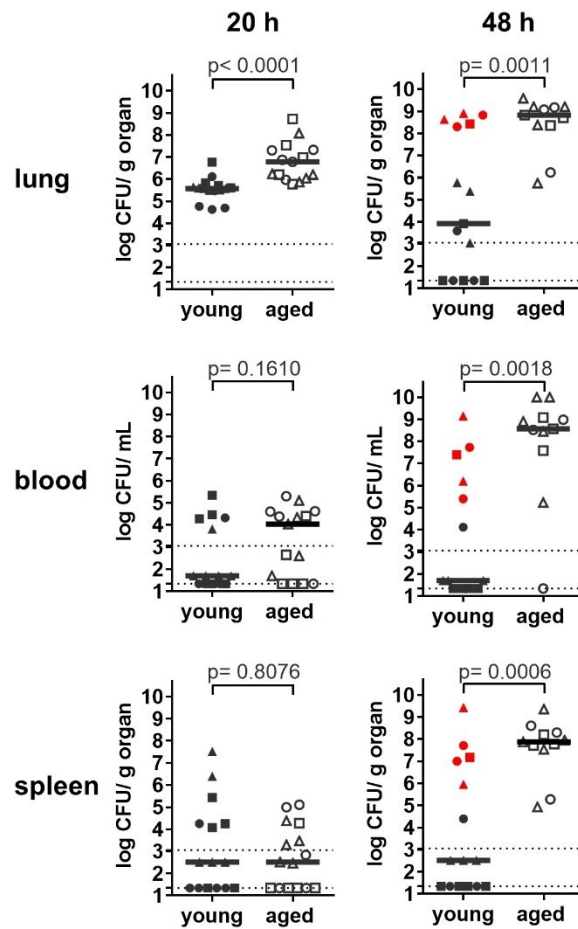


Figure 3. Impaired clearance of pneumococci from the lung and systemic dissemination in aged mice. Bacterial burden in lung, blood and spleen of infected animals were determined at 20 and 48 hours post-infection by CFU plating. Data are derived from the same three independent studies as shown in Fig. 2. (marked by circles, squares and rectangles). Lower limit of detection ranges are marked by dotted lines, bars indicate group median values. Group differences were statistically evaluated using Mann-Whitney U-test; p-values are indicated on the graphs.

Figure 4

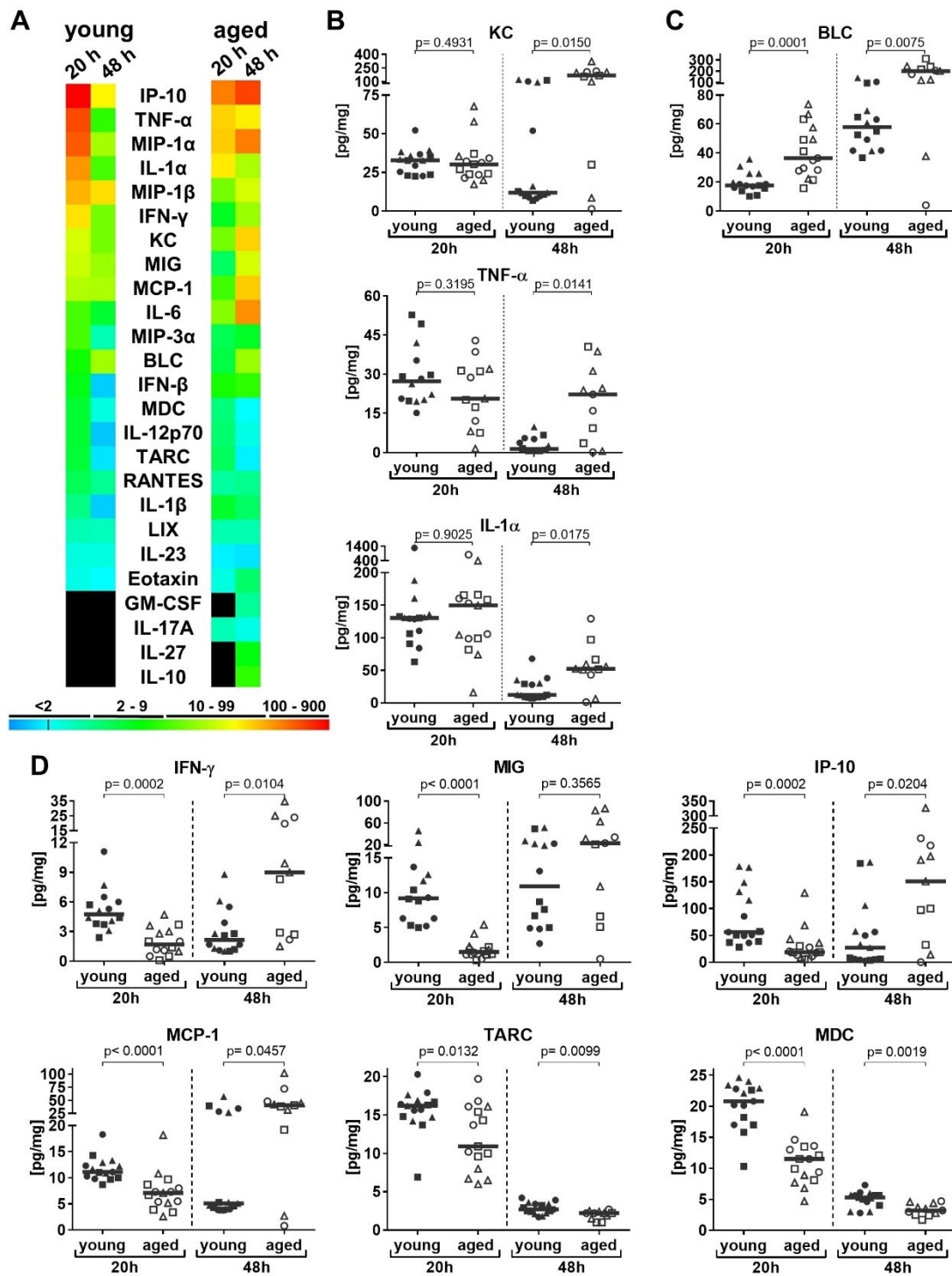


Figure 4. Aged mice display delayed and prolonged inflammatory responses to pneumococcal lung infection. Figure legend see next page.

Figure 4. Aged mice display delayed and prolonged inflammatory responses to pneumococcal lung infection. Levels of pulmonary cytokines and chemokines in aged and young mice were measured at 20 or 48 hours following intranasal infection with 2×10^7 CFU *S. pneumoniae* D39. **A:** Changes in analyte levels calculated relative to the control groups as $\log_2$ (infected value/ mock value). Dynamics in expression levels depicted as heat map with a color scale indicating the median fold changes. Black color indicates median values below the lower limit of detection. **B:** Representative examples for different patterns of changes in analytes in aged and young mice. **C:** Increasing BLC levels in both age groups over time. **D:** Delayed upregulation of IFN- γ and related chemokines in aged mice. Data from the same three independent studies (marked by circles, squares and rectangles) as in Fig. 2 & Fig. 3 are shown, bars indicate group median values. Group differences were statistically evaluated using Mann-Whitney U-test; p-values are indicated on the graphs.

Figure 5

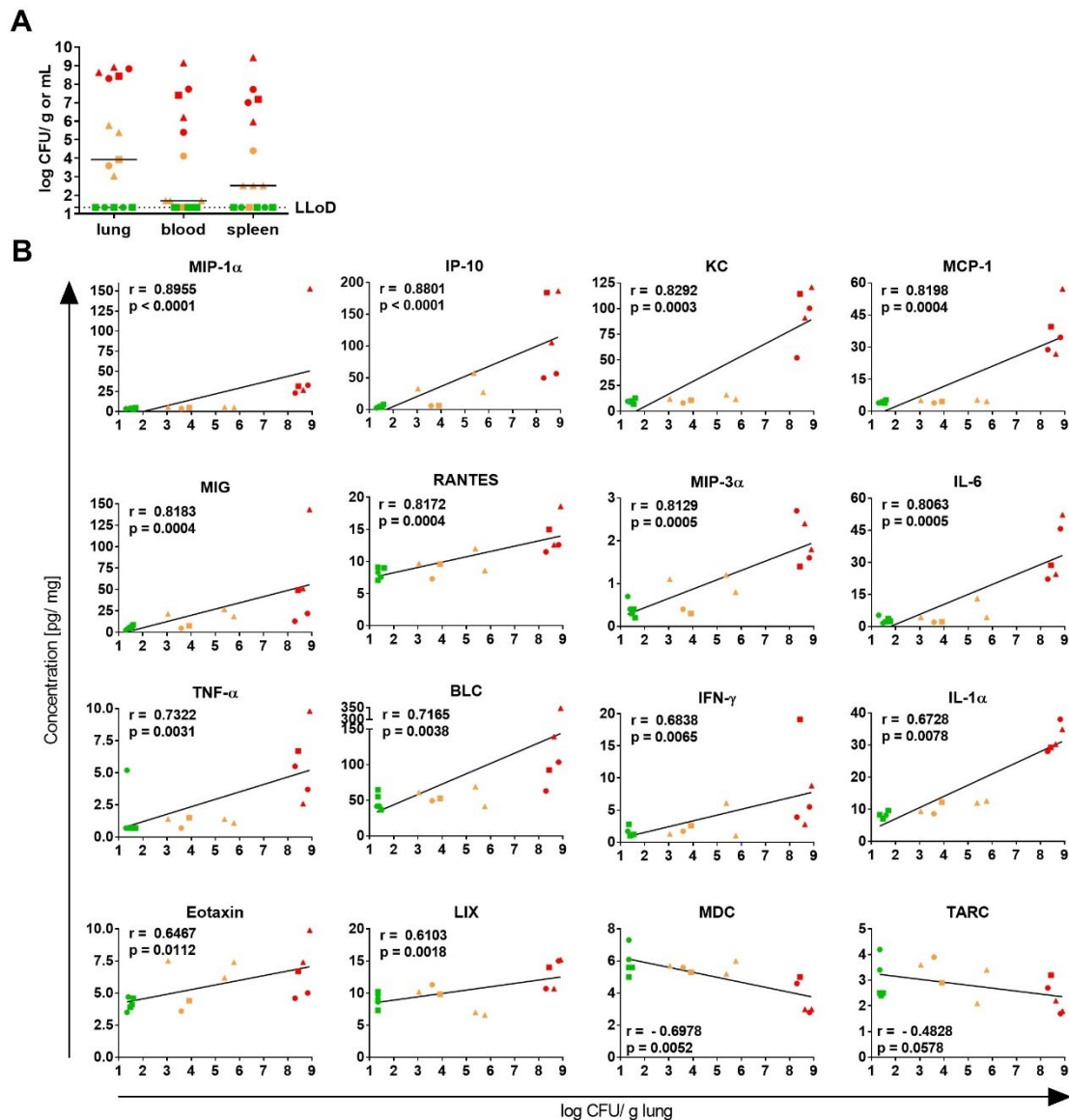


Figure 5. Correlation between bacterial burden and cytokine/chemokine levels in the lungs of young mice infected with *S. pneumoniae*. **A:** Bacterial load in lungs, blood and spleens of young mice at 48 hours post-infection as shown in Fig.3. A subgroup of young mice completely cleared *S. pneumoniae* from the lung (green), showed similar or reduced bacterial burden compared to levels at 20 hours post-infection (yellow), or failed to contain bacterial growth (red) similar to aged mice. **B:** Correlation of inflammatory cytokine/chemokine levels with bacterial lung burden (colors as in panel A) with Spearman coefficients r (two tailed- p values). Analytes not shown were not detectable or did not show correlation.

Table S1

	young	aged	p-value
BLC	2.61 +/- 1.55	10.69 +/- 6.66	< 0.0001
Eotaxin	4.77 +/- 1.49	4.26 +/- 1.42	0.1207
IFN-γ	0.22 +/- 0.06 *	0.44 +/- 0.09	0.0003
IL-1α	1.20 +/- 1.06	1.88 +/- 1.30	< 0.0001
IL-6	1.50 +/- 1.43	1.10 +/- 1.06	0.3010
KC	1.01 +/- 0.73	1.96 +/- 1.50	0.0001
LIX	6.19 +/- 4.19	5.43 +/- 1.72	0.6946
MCP-1	0.65 +/- 0.71	0.81 +/- 0.88	0.2156
MDC	3.70 +/- 0.56	3.45 +/- 0.66	0.0725
MIG	0.51 +/- 0.45 *	0.94 +/- 0.75	0.0039
MIP-1α	0.24 +/- 0.19 *	1.04 +/- 0.82	< 0.0001
MIP-1β	0.48 +/- 0.46 *	1.96 +/- 1.25	< 0.0001
MIP-3α	0.48 +/- 0.28	1.40 +/- 0.62	< 0.0001
RANTES	3.57 +/- 0.94	15.60 +/- 10.65	< 0.0001
TARC	3.20 +/- 0.74	2.78 +/- 1.07	0.0068
TNF-α	0.30 +/- 0.54 *	0.72 +/- 1.26	0.0152

Table S1. Pulmonary levels of inflammatory mediators in young and aged mice. Values of pulmonary inflammatory mediators (pg/mg) in control mice (mock treated) are shown as mean +/- SD. Asterisks indicate that levels of the analyte were close to the lower limit of detection in the majority of young mice and should be regarded with caution. Mediators not shown were could not be detected in the majority of mice. Group differences were statistically evaluated using Mann-Whitney U-test; p-values are indicated on the graphs.

CONCLUDING DISCUSSION

Despite tremendous advances in modern medicine, bacterial infections, including *S. aureus* and *S. pneumoniae*, have remained a serious health concern warranting novel anti-infective approaches.

One such future strategy is passive immunization with monoclonal antibodies to prevent and treat bacterial infections. Inhibition of specific Gram-positive virulence mechanisms rather than direct killing of bacteria seems to be a promising way to support host defences to clear the pathogen. Understanding the underlying molecular mechanisms is crucial to combat staphylococcal and pneumococcal infections.

This dissertation provides novel insights into the virulence mechanisms of two versatile Gram-positive pathogens. The findings highlight the importance of functional redundancy when bacterial virulence factors sharing similar roles in pathogenesis are targeted as anti-infective strategy. The high level of diversity of *S. aureus* and *S. pneumoniae* strains is reflected by the presence of one to multiple genes encoding different virulence factors with redundant function, mutations, variable gene expression affected by media composition *in vitro* etc. These aspects require the assessment of multiple different bacterial isolates to exclude strain-specific differences. This is underlined by the highly divergent leukocidin expression profiles and different contributions of neuraminidases to the total enzymatic activity in diverse clinical isolates observed *in vitro*. These findings are further corroborated by the requirement to synergistically target virulence factors to achieve maximum inhibition that is strain-independent: the combination of monoclonal antibodies ASN-1 (targets *S. aureus* Hla and leukocidins LukSF-PV, LukED, HlgAB and HlgCB), ASN-2 (targets *S. aureus* LukGH), or the combination of two mAbs - #21155 (targets *S. pneumoniae* NanA) and 3C6-H7 (targets *S. pneumoniae* NanB and NanC) - is required to antagonize toxin and neuraminidase functions across a range of clinical *S. aureus* or *S. pneumoniae* isolates, respectively. Cells of the innate and adaptive immune system cannot be fully protected by targeting only a subset of leukocidins (ASN-1 or ASN-2 alone). Similarly, several effects mediated by neuraminidases (e.g. removal of sugars from the murine and human lung epithelium, or binding of pneumococci to human lung tissue) cannot be fully neutralized by only targeting NanA or NanB and/or NanC (mAb #21155 or mAb 3C6-H7 alone).

Although complete blocking of all pneumococcal neuraminidases by the two mAbs seems to be feasible during severe murine pneumococcal pneumonia, host survival, bacterial growth and the extent of inflammation was unaffected. This finding suggests that

pneumococcal neuraminidases do not play essential roles in the lethal murine pneumonia model. A major limitation of this model is the requirement of a high challenge inoculum, which does not reflect human disease progression. Pneumococcal pneumonia usually develops from aspiration of bacteria from the nasopharynx, a step that is circumvented in the murine model applied.

Many bacteria, such as *Pseudomonas aeruginosa*, *Clostridium perfringens* and *Vibrio cholerae* can express neuraminidases. Protein sequence identities between neuraminidases of different species are very low (< 30 %), although conserved residues around the catalytic centers exist. Molecular weights of neuraminidases range from approx. 40 to 120 kDa ¹²³. Neuraminidases of different species can contribute to bacterial sugar acquisition by removing terminal sialic acids from host cells, but reported effects on bacterial virulence vary: blocking of neuraminidases by 2,3-dehydro-2-deoxy-N-acetyl-neuraminic acid (DANA) or addition of anti-GM1 antibodies reduces adherence of *P. aeruginosa* to cystic fibrosis epithelial cells ¹²⁴. Neuraminidase gene deletion mutants of *P. aeruginosa* (Δ 2794) show reduced virulence in mouse models of respiratory but not bloodstream infection ¹²⁵. The two large *C. perfringens* sialidases NanI, NanJ, but not the smaller sialidase NanH can cleave sialic acids from host epithelial cells *in vitro* ¹²⁶. However, gene deletion mutation of both *nanI* and *nanJ* does not affect *C. perfringens* virulence in murine models of gas gangrene. The authors argue that the true role of clostridial neuraminidases during gas gangrene might be masked due to the high bacterial challenge dose required to induce disease in mice ¹²⁷. This resembles the limitation of the lethal murine pneumonia model to address the involvement of *S. pneumoniae* neuraminidases during lung pathogenesis included in this dissertation. Similarly, *Vibrio cholerae* neuraminidase enhances the binding of cholera toxin to murine fibroblast (by unmasking the cholera toxin receptor GM1) *in vitro*, but does not seem to affect virulence *in vivo* ¹²⁸.

Influenza neuraminidase (NA) can also remove terminal sialic acids from the host epithelium. However, influenza NA serves a different biological function compared to bacterial neuraminidases. The removal of sialic acid is required to release new virions from infected host cells to enable viral spread ¹²⁹. Pre-existing antibodies against influenza neuraminidase are associated with increased resistance and are independent correlates of protective immunity in humans ^{130–132}. Notably, a cross-reactive anti-NA antibody that binds to a conserved epitope of 10 NA subtypes across many different lineages can provide hetero-subtypic protection in mouse models ^{133,134}. Several studies have shown that influenza virus infection increases the availability of sialic acid *in vivo*, which in turn favors *S. pneumoniae* growth and adherence to

unmasked host cell receptors. Inhibition of influenza NA (at a time point where viral replication is not affected) protects mice from secondary pneumococcal pneumonia and increases survival^{135,136}. *Vice versa*, *S. pneumoniae* neuraminidases can both promote and inhibit influenza spread from human A549 lung cells if present at low or high concentrations, respectively. Inhibition is achieved by cleavage of α -2,3 and/or α -2,6 linked sialic acids which the virus uses for attachment¹³⁷.

In the influenza field of research, it has been appreciated for decades that *in vivo* models other than mice are needed to improve understanding of human respiratory disease. Ferrets are currently considered as the best model to study influenza lung infections as signs of disease, such as coughing or nasal discharge resemble human symptoms (but are absent in mice)¹³⁸. Moreover, the sialic acid composition of ferret respiratory tracts more closely resembles that of humans. It has recently been demonstrated that murine airways contain mainly terminal N-glycolylneuraminic acid (Neu5Gc), whereas those of both ferrets and humans are decorated by N-acetylneuraminic acid (Neu5Ac). Interestingly, the latter is specifically sensed by *S. pneumoniae* to increase both NanA expression and virulence *in vivo*^{139–141}. Therefore, it is likely that conventional mouse models of pneumococcal airway infection are not ideal predictors of the true contribution of *S. pneumoniae* neuraminidases during human pneumonia pathogenesis. However, species-specific differences in this regard have been largely unexplored.

The situation with *S. aureus* animal models of disease involving leukocidins is completely different. Beyond doubt, different species (such as humans, mice and rabbits) display hugely different susceptibilities towards leukocidins due to differences in host cell receptor repertoire and/ or structure^{41,42}. Improved *in vitro* models are needed to better estimate leukocidin-mediated human pathology. Data included in this dissertation show that LukGH, the most recently discovered *S. aureus* leukocidin, has predominant roles in *S. aureus*-mediated killing of human phagocytic cells *in vitro*. The susceptibility of human polymorphonuclear leukocytes to leukocidin-mediated cytotoxicity greatly differs when stimuli relevant to infection (e.g. bacterial culture supernatant, bacterial proteins and cell wall components but also human IL-8) are present. Different susceptibilities to toxins are mirrored by differences in expression of chemokine and complement receptors, the cognate binding partners of leukocidins. It appears that this phenomenon is not limited to *S. aureus*. This is illustrated with several examples below.

Susceptibility of human cells to Shiga toxin (Stx) from enterohemorrhagic *Escherichia coli* can be markedly increased in presence of inflammatory signals. LPS, tumor necrosis factor

and interleukin-1 can increase susceptibility of human umbilical vein endothelial cells to Stx ¹⁴². The same cytokines increase Stx-mediated cytotoxicity on human brain endothelial cells ^{143,144}. Increased susceptibility reported in these studies was always paralleled by increases in Globotriaosylceramide (Gb3; Stx receptor) induced by inflammatory stimuli.

Actinobacillus actinomycetemcomitans, a Gram negative bacterium that can cause several diseases in humans, including aggressive periodontitis, endocarditis or meningitis, produces a leukotoxin (Ltx) that binds human CD11a/ CD18 (LFA-1), a receptor present on phagocytes. IL-1 β and TNF- α enhance LFA-1 expression on human neutrophil-like HL-60 cells which increases Ltx-mediated apoptosis ¹⁴⁵. Likewise, the main bacterial causative agent of bovine pneumonic pasteurellosis, *Mannheimia (Pasteurella) haemolytica* A1, secretes a leukotoxin (LKT) that specifically targets ruminant leukocytes by binding to the same receptor. The expression of this receptor is greatly increased in presence of IL-1 β , TNF- α , or IFN- γ which in turn enhances LKT binding and cytotoxicity ¹⁴⁶.

Similar to *S. aureus* LukGH, the *Bordetella pertussis* adenylate cyclase toxin CyaA engages CD11b. CyaA is mainly involved in interfering with phagocyte chemotaxis and function, but also induction of apoptosis ^{147,148}. *B. pertussis* produces membrane bound filamentous hemagglutinin (FHA) which is used for bacterial adhesion to the same receptor. Moreover, secreted FHA interacts with a host complex to increase CD11b expression and adhesion ¹⁴⁷. Whether this also leads to altered susceptibility to CyaA (and interference with the recruitment and/or function of immune cells to the site of infection) remains to be shown.

Therefore, beyond the field of *S. aureus* leukocidins, it is important to evaluate potencies of bacterial toxins targeting host cell receptors *in vitro* in a context that more closely mimics inflammatory *in vivo* environments. This is expected to better estimate human host cell susceptibilities, especially when ideal animal models are missing.

As described above, selected stimuli relevant to host inflammation were used to model *S. aureus* leukocidin toxicity on human cells *in vitro*. A different approach was chosen that assessed the interrelation between *S. pneumoniae* virulence and inflammatory signals at the level of the host organism. This helped to elucidate how cytokine and chemokine dynamics during experimental pneumococcal pneumonia differ *in vivo* in an age-dependent manner. Aging in mice and humans is associated with a heightened pro-inflammatory status (“inflammaging”) ^{149,150}, which is thought to be one of the predisposing factors for increased susceptibility to pneumococcal pneumonia ⁵². Data included in this dissertation confirm that aged compared to young mice are more susceptible to pneumococcal pneumonia. Reduced bacterial clearance from the lungs of aged mice was accompanied by delayed induction of

pulmonary IFN- γ and several related chemokines (MIG, IP-10, MCP-1, MDC, TARC) and the general inability to resolve inflammatory responses over time. Young mice on the contrary showed rapid peak inflammatory responses which subsequently diminished. This correlated with improved bacterial clearance from the lungs and reduced invasion of the bloodstream. Studies so far employing aged mouse models to address inflammation during pneumococcal pneumonia have focused on a single time point, which has led to contradictory results. Increased inflammatory responses in young compared to aged mice were reported in studies that measured inflammatory mediators a few hours after infection, whereas the opposite was true if later time points were chosen ^{119–122}. The study included in this dissertation connects these findings and emphasizes that multiple time points are necessary for assessing potential differences in inflammatory responses in aged versus young mouse models of pneumococcal disease.

In summary, this dissertation provides new findings on the interplay between virulence mechanisms of *S. aureus* and *S. pneumoniae* and the host. Signals relevant to the inflammatory milieu *in vivo* greatly modify the susceptibility of human polymorphonuclear leukocytes towards *S. aureus* leukocidins LukSF-PV, LukGH, LukED, HlgAB and HlgCB *in vitro*. Age-related delayed induction of and subsequent failure to resolve inflammation increases *S. pneumoniae* growth and host susceptibility in murine models of severe pneumonia. Functional redundancy of bacterial virulence factors, including *S. aureus* leukocidins and *S. pneumoniae* neuraminidases is a pivotal feature of bacterial pathogens. Therefore, all these aspects require careful consideration when generating novel anti-bacterial agents, such as monoclonal antibodies or protein-based vaccines.

In the future it will be important to further evaluate the involvement of *S. pneumoniae* neuraminidases during human pneumonia. Murine *in vivo* data included in this dissertation could not determine major roles of neuraminidases during lethal pneumonia. However, *in vitro* data generated with human lung cells and primary human lung tissue contradict these findings and clearly demonstrate that mAb-based targeting of neuraminidases can in fact protect human cells from *S. pneumoniae* infection. The mouse model is routinely used to model human disease and offers several advantages including handling, cost and extensive genetic characterization. However, over-interpretation of data from this model should be omitted, especially when addressing obligate human pathogens such as the pneumococcus that have co-evolved with their host over centuries and are highly adapted to thriving in its respiratory tract.

The need of improved *in vitro* and *in vivo* models seems to be more appreciated in the *S. aureus* field of research. This is due to a more profound molecular understanding of species-specific susceptibilities, but also the failure of all clinical vaccine trials hitherto. As shown in this dissertation human immune cells can be clearly protected from *S. aureus*-mediated killing by simultaneous targeting of leukocidins and Hla *in vitro*. Whether these findings translate into improved outcomes of mechanically ventilated patients heavily colonized with *S. aureus* will be ultimately determined by the outcomes of a currently ongoing Phase 2 clinical trial.

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