Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps

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Summary
Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide. Pneumococci can counteract the action of neutrophils with an antiphagocytic capsule and through electrochemical repulsion of antimicrobial peptides via addition of positive charge to the surface. Pneumococci are captured, but not killed in neutrophil extracellular traps (NETs). Here, we study the role of the polysaccharide capsule and lipoteichoic acid (LTA) modification on pneumococcal interaction with NETs. Expression of capsule (serotypes 1, 2, 4 and 9V) significantly reduced trapping by NETs, but was not required for resistance to NET-mediated killing. Pneumococci contain a dlt operon that mediates the incorporation of D-alanine residues into LTAs, thereby introducing positive charge. Genetic inactivation of dltA in non-encapsulated pneumococci rendered the organism sensitive to killing by antimicrobial components present in NETs. However, the encapsulated dltA mutant remained resistant to NET-mediated killing in vitro. Nevertheless, in a murine model of pneumococcal pneumonia, the encapsulated dltA-mutant strain was outcompeted by the wild-type upon invasion into the lungs and bloodstream. This suggests a non-redundant role for LTA alanylation in pneumococcal virulence at the early stage of invasive disease when capsule expression has been shown to be low.

Introduction
The Gram-positive bacterium Streptococcus pneumoniae is a major cause of community-acquired pneumonia, sinusitis and otitis media, but is also an important cause of invasive diseases, such as meningitis and bacteraemia. Even with access to antibiotic treatment and intensive care, pneumococci remain a major cause of morbidity and mortality worldwide (WHO, 2003). An early response of the immune system towards pneumococcal infection is the migration of neutrophils (polymorphonuclear leukocytes) into the infected tissue (Kadioglu and Andrew, 2004). Neutrophils sense inflammatory mediators, such as IL-8, released by pulmonary epithelial cells, and transmigrate from the bloodstream to the site of infection where they start up a plethora of bacterial killing mechanisms. Neutrophils recognize bacteria and kill them by secretion of antimicrobial peptides (AMPs) and proteases and by engulfment into a phagosome, containing reactive oxygen species, enzymes and AMPs (Weinrauch et al., 2002; Mayer-Scholl et al., 2004; Nathan, 2006). Pneumococci, however, have developed a wide array of resistance mechanisms against neutrophils and AMPs. Most pneumococcal strains isolated from the bloodstream of patients with invasive pneumococcal disease produce a thick capsular polysaccharide of which more than 90 different types can be distinguished. The capsule confers protection from professional phagocytes such as neutrophils (Cross, 1990) and it has been suggested that the capsular type affects the ability to cause invasive disease (Brueggemann and Spratt, 2003; Sandgren et al., 2005; Sjostrom et al., 2006). Resistance to AMPs is also a widely observed phenomenon in Gram-positives (Peschel, 2002). Because of the cationic nature of most AMPs, electrostatic attraction to the negatively charged bacterial cell envelope is hypothesized to be essential for AMPs to work (Epand and Vogel, 1999). Consequently, changes in the bacterial surface, especially the introduction of positive charge is a way to repel AMPs (Kristian et al., 2005). Positive charge can be introduced by D-alanylation of...
lipoteichoic acids (LTAs) – polymers anchored to the cytoplasmic membrane via glycolipids. This process involves a D-alanine-activating enzyme and a D-alanine-D-alanyl carrier protein ligase (Chevion et al., 1974). The respective genes are encoded on the dlt operon (Heaton and Neuhaus, 1992; Perego et al., 1995; Kristian et al., 2005), which is also present in the two sequenced S. pneumoniae isolates TIGR4 and R6 (Hoskins et al., 2001; Tettelin et al., 2001). However, because of a point-mutation in the dlt operon, DltA is non-functional in R6 (Kovacs et al., 2006). Deletion of the dltA gene in S. pneumoniae leads to decreased resistance to AMPs and in group A streptococci (GAS) also to decreased survival in neutrophils (Kristian et al., 2005; Kovacs et al., 2006).

Pneumococci colonize the nasopharynx of up to 60–70% of all preschool children (Garcia-Rodriguez and Fresnadillo Martinez, 2002; Masuda et al., 2002; Henriques-Normark et al., 2003; Nunes et al., 2005) but only rarely cause invasive pneumococcal disease, pointing to the presence of uncharacterized host defence mechanisms. We recently proposed a mechanism for the confinement of pneumococci to the site of infection by neutrophil extracellular traps (NETs) (Beiter et al., 2006). NETs are formed upon stimulation of neutrophils and have been shown to bind, disarm and kill pathogens extracellularly (Brinkmann et al., 2004; Urban et al., 2006). They constitute an extracellular fibrous web of DNA, histones and granule enzymes such as neutrophil elastase (NE) and myeloperoxidase (MPO). Killing is mediated by AMPs such as bactericidal permeability inducing factor (BPI), defensins and even histones (Hirsch, 1958). However, encapsulated, virulent pneumococci are not killed by the antimicrobial activity present in NETs (Beiter et al., 2006). Nevertheless, NET trapping seems to be a host mechanism to confine the infection, and prevent systemic bacterial spread. However, pneumococci can escape from NETs and regain the ability to spread through the action of a surface-bound DNase that leads to the destruction of the DNA trap-backbone (Beiter et al., 2006).

The level of NET trapping, the sensitivity level to antimicrobial components and the ability to degrade NETs are all likely parameters that affect whether a pneumococcal infection will result in severe invasive disease. Here we studied the role of the polysaccharide capsule and LTA modification on NET-interaction in vitro as well as on virulence in vivo. We found that capsule expression reduced trapping, and that capsule in concert with D-alanylation of LTA contributed to the resistance to NET killing in vitro. In encapsulated pneumococci D-alanylation of LTA promoted spread to the lungs and to the bloodstream. This suggests a critical role for LTA alanylation in resisting host defence mechanisms during invasive pneumococcal disease.

**Results**

The pneumococcal polysaccharide capsule protects from NET trapping but is not required for resistance to NET-mediated killing

We tested the role of the pneumococcal polysaccharide capsule on the interaction with NETs and neutrophils. For this, sets of encapsulated (serotypes 1, 2, 4 and 9V) and corresponding non-encapsulated strains (serotypes xR, see Experimental procedures) were used. To investigate the trapping of pneumococci by NETs, human neutrophils were activated to make NETs, infected with FITC-labelled pneumococci and stained for different NET markers in vitro (Fig. 1A). NETs were identified as filamentous structures that contain the markers DNA (blue) and NE (red). Pneumococci were stained in green. Both encapsulated (Fig. 1A, left column) and non-encapsulated pneumococci (right column) were captured in NETs. However, substantially higher numbers of non-encapsulated pneumococci were trapped in NETs compared with encapsulated bacteria. To numerically compare the extent of trapping, we determined the number of bacteria per micrometre of NET structure in 10 randomly recorded images of filamentous NETs for each strain (Fig. 1B). To verify and to more exactly quantify the percentage of bacteria trapped by NETs, we chose a colony-forming units (cfu)-based assay (Fig. 1C). For this, we compared the number of cfu in neutrophil cultures with intact NETs, and cultures with NETs degraded by bovine pancreatic DNase, as described in Experimental procedures. Both quantifications indicated that NETs trapped non-encapsulated pneumococci (types xR) to a significantly higher extent than encapsulated ones ($P \leq 0.001$, each) (Fig 1B and C). The increase ranged between fourfold (serotype 4) and 12-fold (serotype 1). This strongly suggests that the polysaccharide capsule limits trapping of pneumococci by NETs.

No significant differences in NET trapping could be observed between all studied encapsulated strains. Also, strains with charged (types 1, 2, 4 and 9V) and non-charged (types 7F and 14) capsular polysaccharides were trapped to the same extent (data not shown).

We next analysed the effect of the pneumococcal polysaccharide capsule on the sensitivity towards killing by NETs (Fig. 2). To distinguish between non-NET-mediated killing, such as phagocytosis (white) and NET bactericidal activity (black), phagocytosis was blocked by the actin polymerization inhibitor Cytochalasin D (Brenner and Korn, 1979; Staali et al., 2003; Brinkmann et al., 2004). The concentration used, efficiently blocked phagocytosis for all used strains (Fig. S1) while not interfering with the bacteria–NET interaction (Fig. S2). Thirty-minute incubation was allowed for killing and phagocytosis to take place. Encapsulated pneumococci were highly
Fig. 1. The pneumococcal polysaccharide capsule reduces trapping by NETs in vitro.

A. Neutrophils were stimulated for NET formation and infected with encapsulated and non-encapsulated pneumococci (of serotypes 1, 2, 4 and 9V) at a multiplicity of infection (moi) of 10. The samples were fixed 5 min post infection and stained for DNA (blue) and neutrophil elastase (red). *S. pneumoniae* were labelled with FITC before infection (green). Scale bars represent 20 μm. NETs were identified as filamentous structures. Pneumococci were bound to the NET structures; however, the encapsulated strains were trapped to a lower extent than their non-encapsulated derivatives.

B. The number of bacteria per micrometre of NET filament was determined for the strains used in A. Mean values and standard deviations (SD) are shown. Trapped bacteria in pictures with filamentous NET structures were counted manually and the NET filament length was determined as shown in the two immunofluorescence pictures. The non-parametric Mann–Whitney test was used for statistical comparison between encapsulated and non-encapsulated strains (*P* < 0.001, each).

C. Neutrophils were stimulated for NET formation and infected with pneumococcal strains at an moi of 10. The percentage of cfu trapped in NETs 5 min post infection is shown for encapsulated pneumococci (type 1, 2, 4 and 9V) and their capsular knockouts (types xR). Mean values and standard deviations (SD) of per cent bacterial trapping are shown. The percentage of trapping was determined with the help of bovine pancreatic DNase that degrades NETs, thereby freeing previously trapped bacteria. Assays were performed at least three times for each strain and the non-parametric Mann–Whitney test was used for statistical comparison between encapsulated and non-encapsulated strains (*P* < 0.001, each). Non-encapsulated pneumococci were trapped significantly higher than their encapsulated counterparts. ***, *P* < 0.001; **, *P* < 0.01; *, *P* = 0.001.
resistant both to NET-mediated killing (black) and to non-NET-mediated killing by neutrophils (Fig. 2). This applied to both charged (types 1, 2, 4 and 9V) and non-charged (types 7F and 14) serotypes (data not shown). All non-encapsulated pneumococci (types xR) were killed to 25–64% through non-NET-mediated killing, such as phagocytosis. However, except for type 2R (R6), no pronounced bactericidal effect of NETs could be observed. Hence, pneumococci were resistant to NET-mediated killing to a high extent. Resistance, however, was not conferred by the capsule alone.

D-alanylation of LTAs does not affect NET trapping but reduces sensitivity to killing by NETs and AMPs

The encapsulated type 2 strain D39 was recently shown to express D-alanylated LTA in contrast to the non-encapsulated type 2 derivative R6, which contains an inactivating mutation in \( dltA \) (Kovacs et al., 2006). The percentage of NET trapping of wild-type and \( dltA \) mutant strains was determined in the presence or absence of capsule expression (Fig. 3). To test this, we generated \( dltA \)-mutants in TIGR4 and TIGR4R (TIGR4\( \Delta dltA \), and TIGR4R\( \Delta dltA \)). The encapsulated strains (TIGR4, TIGR4\( \Delta dltA \) and D39) were trapped to the same, low extent of 7–16%. This is in contrast to the high extent of trapping (54–69%) observed for the non-encapsulated strains (TIGR4R, TIGR4\( \Delta dltA \) and R6). When comparing \( dltA \) mutants with their parental strains, a statistically non-significant increase of trapping was observed in this cfu-based assay \((P = 0.09–0.18)\). When counting the numbers of bacteria visually trapped in NET structures, a similar result was obtained (Fig. S2). Hence, the data showed that D-alanylation of LTA does not significantly affect trapping \( \text{in vitro} \). Genetic inactivation of choline binding protein A (CbpA), a choline binding protein associated with teichoic acids as well as LTA (Rosenow et al., 1997), did not affect NET trapping (Fig. 3, Fig. S2). Thus, NET-trapping is primarily determined by the presence or absence of capsule.

Next we analysed the effect of LTA D-alanylation on sensitivity to NET killing (Fig. 4). The R6 strain with a non-functional DltA enzyme was killed by NETs up to 12.8%. Deletion of \( dltA \) in the encapsulated TIGR4 (TIGR4\( \Delta dltA \)) did not show an increased sensitivity to NET killing in contrast to the non-encapsulated TIGR4R mutant (TIGR4R\( \Delta dltA \)), where a marked increase in sensitivity to NETs was observed. All non-encapsulated strains, irrespective of LTA D-alanylation, were also killed by non-NET-mediated killing, such as phagocytosis. Absence of CbpA in both encapsulated and non-encapsulated background did not increase NET-killing (Fig. 4). We can then conclude that D-alanylation of LTA in combination with capsule expression causes pneumococcal resistance to NET-mediated killing.

D-alanylation of lipoteichoic acids does not affect NET trapping. Neutrophils were stimulated for NET formation and infected with different pneumococcal strains at an moi of 10. The percentage of cfu trapped in NETs 5 min post infection is shown for the encapsulated pneumococci TIGR4 and D39, the non-encapsulated strains TIGR4R and R6 as well as \( dltA \) and \( cbpA \) mutants in the TIGR4 and TIGR4R background. The non-encapsulated R6 strain is a spontaneous \( dltA \)-mutant. Mean values and standard deviations (SD) of per cent bacterial trapping are shown. The percentage of trapping was determined with the help of bovine pancreatic DNase that degrades NETs, thereby freeing trapped bacteria. Assays were performed at least three times for each strain and analysed with the non-parametric Mann–Whitney test. Knockout of \( dltA \) or \( cbpA \), the control, did not lead to increased trapping by NETs.

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LTA D-alanylation of encapsulated TIGR4 is important for virulence in systemic infection

To determine the role of capsule and D-alanylated LTA in vivo, we infected C57BL/6 mice intranasally with encapsulated TIGR4 and TIGR4ΔdltA in a 1:1 ratio in a competition experiment. Then the bacterial load was determined in the upper respiratory tract (URT), the lungs and the bloodstream. Figure 5 shows that in mice with pneumonia, twice as many TIGR4 as TIGR4ΔdltA were recovered from the URT (CI = 0.49). This indicates a slight disadvantage for the mutant in the URT, as in vitro both strains show the same growth rates (data not shown). In the lungs of mice with pneumonia, seven times more TIGR4 than TIGR4ΔdltA bacteria were recovered (CI = 0.15) (Fig. 5). Already, 24 h post infection (p.i.) seven times more TIGR4 than mutant bacteria were found in the bloodstream (CI = 0.15). This difference was even more pronounced at 48 h p.i. when 14 times as many TIGR4 as mutant bacteria were found (CI = 0.07). Most significantly, 11 times more wild-type than mutant bacteria were recovered from blood in mice with pneumonia (CI = 0.09). These data strongly suggest that TIGR4ΔdltA is less efficient in reaching and propagating in the lungs and in the bloodstream, implying an important role for LTA D-alanylation in encapsulated pneumococci.

Discussion

Pneumococci use the nasopharynx of humans as their sole ecological niche. Even though pneumococci kill approximately 1–2 million children annually, disease is a rare event considering that this organism colonizes the nasopharynx of most children, at some point in time (Masuda et al., 2002). Innate immune responses via Toll-like receptors play an important role in confining this potentially devastating pathogen to the URT (Albiger et al., 2005). Resident macrophages, infiltrating neutrophils and secreted AMPs are important mediators of bacterial eradication in the lower respiratory tract. In pneumococcal pneumonia large numbers of neutrophils infiltrate the lung tissue. Mice strains, deficient in recruitment and/or function of neutrophils have been shown to be more susceptible to pneumococci (Gingles et al., 2001; Partida-Sanchez et al., 2001). Moreover, in humans with congenital neutropenia respiratory infections are common (Rezaei et al., 2005).

Most pneumococcal isolates from healthy carriers and patients with invasive disease express capsular polysaccharide of which 90 different serotypes have been described. The capsule is important both for causing invasive disease and for colonizing the upper airways. Expression of capsule, however, is a two-edged sword for...
the pneumococcus. On the one hand, it prevents phagocytosis by neutrophils and macrophages, on the other hand, it may hinder adhesion and subsequent invasion of the lung. Most pneumococcal capsules are highly hydrated and contain numerous anionic charged sites that could negatively affect adhesion to cells. The recent discovery of an adhesive pneumococcal pilus provides one explanation why encapsulated pneumococci are capable of adhering to respiratory epithelial cells (Barocchi et al., 2006). It has also been demonstrated that pneumococci may undergo a phase variation where transparent, less encapsulated variants are found in the bloodstream (Weiser et al., 1994; Kim and Weiser, 1998). In addition, capsule expression may be downregulated as a consequence of bacterial interactions with respiratory cells (Hammerschmidt et al., 2005).

Another pneumococcal surface-exposed polymer is LTA, which is anchored to the cytoplasmic membrane via glycolipids. For other Gram-positive pathogens such as Staphylococcus aureus, as well as Group A and Group B streptococci, D-alanylation of LTA mediated by the dlt operon incorporates positive charge into the bacterial surface (Poyart et al., 2003; Kristian et al., 2005; Hunt et al., 2006; Koprivnjak et al., 2006). The dlt operon is also present in the genome of TIGR4 (Tettelin et al., 2001). Recently, it was established that the dlt operon in pneumococci allows D-alanylation of LTA, and that a mutation in one of the genes, dltA, increases susceptibility to the AMPs nisin and gallidermin (Kovacs et al., 2006). In addition, Kovacs et al. demonstrated that the commonly used non-encapsulated strain R6 is carrying a dltA mutation abolishing D-alanylation. Hence, pneumococci express at least two different surface macromolecules, capsular polysaccharide and D-alanylated LTA, that both are likely to affect the interaction with host cells and effector molecules of the innate immune system.

We have previously demonstrated, using a murine model for pneumococcal pneumonia, that NETs are generated from infiltrating neutrophils. NETs were capable of trapping pneumococci in vitro. As demonstrated for pneumococci and GAS, bacterial degradation of the NET DNA backbone allows bacteria to free themselves, thereby facilitating spread (Beiter et al., 2006; Buchanan et al., 2006). We show in this paper that the degree of NET trapping is considerably reduced in encapsulated pneumococci as compared with their non-encapsulated mutants (Fig. 1). Trapping, however, was not affected whether or not LTA was D-alanylated or the surface-located CbpA was expressed or not (Fig. 3). It is unlikely that the anionic nature of most pneumococcal capsules is the sole reason for NET-evasion, because isolates expressing neutral capsules (serotypes 7F and 14) also exhibited a low NET trapping (data not shown). The relative impact of reduced NET trapping for pneumococcal virulence has not been possible to unanimously characterize in vivo, as the capsule also mediates resistance to neutrophil phagocytosis. However, NET evasion clearly constitutes a bacterial virulence factor as evidenced by the discovery of NET-degrading DNase activity in GAS and S. pneumoniae (Aziz et al., 2004; Sumby et al., 2005; Beiter et al., 2006; Buchanan et al., 2006). Hence, these publications indirectly pointed to the importance of NETs in the pathogenesis of streptococcal infections such as pneumonia and necrotizing fasciitis.

Unlike Shigella flexneri and other pathogens (Brinkmann et al., 2004; Urban et al., 2006), pneumococci are not killed by the antimicrobial components present in NETs (Beiter et al., 2006). Here we demonstrate that pneumococci lacking both capsule expression and D-alanylation of LTA become sensitive to NET killing in vitro (Fig. 4). The exact mechanism for this cooperative effect is not known. Another cooperative effect involving the capsule is observed for bacterial lysis. Pneumococci show the ability of autolysis, mediated by the murein hydrolase LytA (Sanchez-Puelles et al., 1986). We have previously demonstrated that loss of the pneumococcal capsule enhances LytA-mediated autolysis upon reaching the stationary phase (Fernebro et al., 2004). Inactivation of the dltD gene in Lactococcus lactis, a non-encapsulated pathogen, also increased autolysis (Steen et al., 2005). We have evidence that the here used non-encapsulated dltA mutant lyses faster in stationary phase as compared with its parental strain (J. Ries et al, unpubl. data). This is not the case for the encapsulated dltA mutant. Hence, a cooperative effect between capsule and D-alanylated LTA can be established for LytA-mediated pneumococcal autolysis, as well. One clue might be the observation that increased negative surface charge (as in DltA) leads to more efficient binding of autolysins (Wecke et al., 1996). Whether or not the negative effect of D-alanylated LTA and capsule on the murein hydrolase LytA plays a role in pneumococcal resistance or pathogenesis remains to be determined.

Using a signature-tagged mutagenesis approach in TIGR4, dlt genes were identified as virulence genes (Hava and Camilli, 2002). Here we demonstrate that an encapsulated dltA mutant of TIGR4 exhibits a slightly decreased ability to colonize the URT as compared with its LTA D-alanylated parental strain (Fig. 5). Once bacteria spread to the lungs and the bloodstream D-alanylation of LTA clearly provides a marked advantage. It is tempting to suggest that this advantage is due to a reduced clearance during the initial phase of pneumonia and invasive disease, when capsule expression is believed to be reduced (Hammerschmidt et al., 2005). During this early phase of infection, resistance to cationic peptides present in NETs and in the circulation may be particularly depen-
dent upon the positive surface charge provided by D-alanylated LTA.

**Experimental procedures**

**Bacterial strains used**

Pneumococci were grown on blood agar plates over night at 37°C in 5% CO₂. The bacteria were subcultured in semi-synthetic medium c + y (Lacks and Hotchkiss, 1960) to an OD₆₀₀ of 0.25 and diluted in PBS if needed. The following strains were used: a clinical isolate of serotype 1 (PJ1354), the serotype 2 strain D39, and the serotype 4 strain TIGR4 (Tettelin et al., 2001) and a clinical isolate of serotype 9V (I95) (Fernebro et al., 2004), as well as their non-encapsulated derivatives, type 1R (see below), type 2R (R6) (Hoskins et al., 2001), type 4R (TIGR4R) (Fernebro et al., 2004) and type 9VR (Fernebro et al., 2004).

We used polymerase chain reaction (PCR) ligation mutagenesis (Sung et al., 2001; Lau et al., 2002) to knockout dltA or cbpA in TIGR4 and TIGR4R and to knock out the capsule operon in the serotype 1 strain. For dltA, primers used for construction and screening of deletion alleles were 5'-TCCATAGGCGATATAGTACC-3' and 5'-TGGGCCCCAGCT TCTCTTTTTAGGCC-3' flanked with Apal for the upstream fragment and 5'-TTGGATCTGACACAATAGGGATTCTCC-3' and 5'-CTTCCGTGTCTATTAGAACC-3' flanked with BamHI for the downstream fragment. For cbpA, the following primers were used: 5'-ATGATTTAGCGGATTATTTATGAACCT-3' and 5'-TTGGGGCCCACTTTACTAAATCTCTCTATATGAA-3' flanked with Apal for the upstream fragment and 5'-ATGATTTAGCGGATTATTTATGAACCT-3' and 5'-TTGGATCTGACACAATAGGGATTCTCC-3' and 5'-CTTCCGTGTCTATTAGAACC-3' flanked with BamHI for the downstream fragment. For knocking out the capsule operon, the following primers were used: 5'-AGAGAGCCTTTTTAGTGGC-3' and 5'-TTTTGGGGCCCACTTTACTAAATCTCTCTATATGAA-3' flanked with Apal for the upstream fragment and 5'-ACCATGACCTCCTTGATCTCGG AGTTGGCCAGC-3' flanked with BamHI for the downstream fragment. For knocking out the capsule operon, the following primers were used: 5'-AGAGAGCCTTTTTAGTGGC-3' and 5'-TTTTGGGGCCCACTTTACTAAATCTCTCTATATGAA-3' flanked with Apal for the upstream fragment and 5'-ACCATGACCTCCTTGATCTCGG AGTTGGCCAGC-3' flanked with BamHI for the downstream fragment. The PCR products were ligated to an erythromycin cassette (GenBank: AB057644) (for dltA and cbpA knockouts) or to a kanamycin cassette (Janus) (capsule knockout), containing Apal and BamHI sites and transformed into the recipient pneumococcal strain (TIGR4, TIGR4R or serotype 1) as previously described (Bricker and Camilli, 1999). The resulting transformants were selected on blood agar plates containing erythromycin (1 mg ml⁻¹) or kanamycin (400 mg ml⁻¹) and confirmed by PCR and sequencing of the insertion area.

**Isolation of neutrophils from peripheral blood**

Neutrophils were isolated from heparinized human peripheral blood, taken from healthy donors as approved by the local ethical committee for experiments involving humans. We sedimented the blood in 3% dextran (MP Biomedicals), performed a density centrifugation through HyPaq ficoll (Amersham) and collected the pellet. After washing and hypotonic lysis the cells were counted and seeded in RPMI (Sigma) containing 10 mM Hepes (Sigma) and 2% human serum albumin (HSA) (Grifols) on surfaces treated with 0.001% poly-L-lysine (Sigma) to foster adherence of the cells (Brinkmann et al., 2004).

**Quantification of trapping** was also performed by seeding neutrophils and activating them with 25 nM PMA for 10 min. After washing, treatment with 10 μg ml⁻¹ Cytochalasin D (as above) inhibited phagocytosis. An moi of 10 was used for infection with bacteria grown to OD₆₀₀ of 0.25. After brief centrifugation and 5 min incubation, the supernatant was removed and used for serial plating to quantify viable bacteria. The cells were subsequently treated for 15 min with RPMI ± bovine pancreatic DNase (In Vitro) to dissolve NETs. The DNase concentration of 20 U ml⁻¹ did not affect pneumococcal viability, yet dissolved NETs. Viable bacteria were quantified by plating of serial dilutions. The percentage of cfu trapped in NETs was calculated according to the formula: %trapped = [(cfuDNase – cfuRPMI)/(cfuDNase + cfuDNase+cfuDNase)] x 100.

**Analysis of phagocytosis and inhibition with Cytochalasin D**

Neutrophils were activated with 25 nM PMA for 10 min and then washed. RPMI (±10 mM Hepes, 2% heat-inactivated human serum) with or without 10 μg ml⁻¹ Cytochalasin D (to block phagocytosis) was added to the samples for 15 min. TIGR4(R) pneumococci were grown to an OD₆₀₀ of 0.25 and labelled as described below. An moi of 10 was used for infection of the neutrophil cultures, followed by brief centrifugation. After 30 min incubation time, cells were fixed with 4% paraformaldehyde (PFA). Immunofluorescence stainings were performed as outlined below. The extent of trapping was analysed shortly (5 min) after bacterial addition to the neutrophils in order to avoid interfering effects such as killing by NETs, AMPs or by phagocytosis and through the effect of the pneumococcal DNase that is degrading NETs (Beiter et al., 2006).

The number of trapped bacteria in these samples was analysed in a way to derive ‘bacteria per micrometre of NET filament’. For this, 10 images of filamentous NET structures were taken for each strain in a blind manner. NET-associated bacteria were counted manually. The NET filament length was determined with the Zeiss software LSM Image Browser as shown in the two immunofluorescence pictures of Fig. 1B. The length of individual arms/branches was summed up.

**Neutrophil killing assays**

Neutrophils were seeded, activated with 25 nM PMA for 10 min and washed. Control samples did not contain neutrophils. RPMI
Samples were analysed using a Zeiss META LSM confocal microscope. With 9% Mowiol 4–88 (Calbiochem) in glycerol-Tris (1:4, v/v), added 1:1000 in ultra pure water, before samples were mounted (mutant input/wild-type input). The minimal bacterial count was the theoretical detection limit (1000 for cfu ml⁻¹). The following primary and secondary antibodies were used: rabbit-α-human neutrophil elastase (Calbiochem), AlexaFluor488-labelled goat-α-FITC (Molecular Probes) and Cy3-conjugated goat-α-rabbit (Jackson). Draq5 (Biostatus) was added 1:1000 in ultra pure water, before samples were mounted with 9% Mowiol 4–88 (Calbiochem) in glycerol-Tris (1:4, v/v).

Samples were analysed using a Zeiss META LSM confocal microscope.

Mice

For competition experiments, 6- to 8-week-old female C57BL/6 mice (bred in own animal facility) were anaesthetized with isoflurane (Forenec, Abbott) and infected intranasally with wild-type and mutant strain in equal amounts, -5 x 10⁶ cfu per strain and mouse (in a volume of 20 µl suspension). The mice health status was monitored according to the following scores: 0 = healthy, 1 = piloerection, 2 = reduced motility, 3 = fever, more pronounced reduced motility, 4 = 1, 2, 3 more pronounced and 5 = moribund. Mice were sacrificed when they reached score ≥ 3 or at 168 h p.i. Viable bacteria were quantified by serial plating of blood samples (5 µl) at 24 h p.i., 48 h p.i. and when the animals were sacrificed.

After removal, the right lungs were weighed and homogenized in PBS and used for the quantification of viable bacteria. To determine the number of bacteria in the URT we performed nasopharyngeal-tracheal lavages post-mortem. Samples recovered from mixed infections were plated on unsupplemented blood agar plates and on blood agar plates containing 1 µg ml⁻¹ erythromycin. The competitive index (CI) was calculated according to the formula: CI = (mutant vs wild-type output)/(mutant input/wild-type input). The minimal bacterial count was the theoretical detection limit (1000 for cfu ml⁻¹ blood and 1 for cfu mg⁻¹ lung).

The non-parametric Mann–Whitney test was used for all assays, except for the comparison between with and without Cytochalasin D treatment in Fig. S2, where the paired t-test was used. Statistical significance was defined as a P-value of < 0.05.

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S. pneumoniae resistance against neutrophil traps

Statistical analyses

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Only non-encapsulated pneumococci are phagocytosed by polymorphonuclear leukocytes (PMNs), a process that can be efficiently blocked by Cytochalasin D. A. Neutrophils were stimulated for NET formation and infected with encapsulated and non-encapsulated TIGR4 pneumococci at an moi of 10. Half of the samples were treated with 10 μg ml⁻¹ Cytochalasin D to block phagocytosis. Thirty minutes post infection, the samples were fixed and stained for DNA (blue) and neutrophil elastase (red). S. pneumoniae were labelled with FITC before addition (green). Scale bars represent 20 μm. Phagocytosis of non-encapsulated bacteria (shown in inset) was observed in the absence of Cytochalasin D. Addition of Cytochalasin D inhibited this process efficiently. The mean numbers of bacteria (plus standard deviations) per cell were quantified for each strain in the absence (B) or presence (C) of Cytochalasin D. The non-parametric Mann–Whitney test was used for statistical comparison between encapsulated and non-encapsulated strains.

Fig. S2. Phagocytosis inhibition by Cytochalasin D does not influence bacteria–NET interaction. The number of bacteria per micrometre of NET filament 5 min p.i. was determined in the presence (white) or absence (black) of 10 μg ml⁻¹ Cytochalasin D. Mean values and standard deviations (SD) are shown. Trapped bacteria in pictures with filamentous NET structures were counted manually and the NET filament length was determined with the Zeiss LSM Image Browser software. The paired t-test was used for statistical comparison between with and without Cytochalasin D treatment for each strain. Cytochalasin D treatment did not affect the interaction of pneumococci with NETs in terms of trapping.

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