Nod1 Signaling Overcomes Resistance of \textit{S. pneumoniae} to Opsonophagocytic Killing

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Airway infection by the Gram-positive pathogen \textit{Streptococcus pneumoniae} (Sp) leads to recruitment of neutrophils but limited bacterial killing by these cells. Co-colonization by Sp and a Gram-negative species, \textit{Haemophilus influenzae} (Hi), provides sufficient stimulus to induce neutrophil and complement-mediated clearance of Sp from the mucosal surface in a murine model. Products from Hi, but not Sp, also promote killing of Sp by \textit{ex vivo} neutrophil-enriched peritoneal exudate cells. Here we identify the stimulus from Hi as its peptidoglycan. Enhancement of opsonophagocytic killing was facilitated by signaling through nucleotide-binding oligomerization domain-1 (Nod1), which is involved in recognition of γ-D-glutamyl-meso-diaminopimelic acid (meso-DAP) contained in cell walls of Hi but not Sp. Neutrophils from mice treated with Hi or compounds containing meso-DAP, including synthetic peptidoglycan fragments, showed increased Sp killing in a Nod1-dependent manner. Moreover, Nod1\textsuperscript{1−/−} mice showed reduced Hi-induced clearance of Sp during co-colonization. These observations offer insight into mechanisms of microbial competition and demonstrate the importance of Nod1 in neutrophil-mediated clearance of bacteria in vivo.

Introduction

Successful pathogens have mechanisms both to avoid triggering inflammatory responses and/or to evade the inflammatory response they induce in their host. In the case of the Gram-positive \textit{Streptococcus pneumoniae} (Sp), a major pathogen of the human respiratory tract, infection involving normally sterile parts of the airway is characterized by acute inflammation with a marked and brisk recruitment of neutrophils [1]. This neutrophil influx, however, is often insufficient to clear the infection until type-specific antibody promotes opsonophagocytic killing. Before such antibody is generated, pneumococci are relatively resistant to neutrophil-mediated killing even when opsonized by complement [2]. The inability of phagocytes to eliminate pneumococci in this period may account for the rapid and often overwhelming progression of pneumococcal pneumonia, a disease responsible for more than a million deaths a year [3]. In fact, in experimental acute pneumonia, neutrophils enhance the likelihood of death without impacting bacterial clearance [4]. Likewise, in a murine model of carriage, intranasal inoculation of Sp induces recruitment of neutrophils into the nasal spaces, yet systemic depletion of neutrophils has little effect on the density of colonizing bacteria [5,6]. In contrast, when co-colonized with the Gram-negative respiratory tract bacteria \textit{Haemophilus influenzae} (Hi), the neutrophil influx is sufficient to rapidly clear Sp from the mucosal surface [6]. Clearance during co-colonization is not seen if either neutrophils or complement are systemically depleted, indicating that killing occurs through neutrophil-mediated phagocytosis of Sp opsonized by complement. These in vivo observations demonstrate that one microbe can co-opt the innate immune response of the host to prevail over a competitor that resides within a similar niche. Enhanced killing of Sp can be modeled \textit{ex vivo} using neutrophils derived from peritoneal exudates cells (PECs) treated in vivo with Hi or its products. Thus, components of Hi are sufficient to stimulate neutrophil activity that overcomes the resistance of complement-opsonized Sp to phagocytic killing.

The focus of this study is to define the mechanism leading to effective neutrophil-mediated killing of Sp that occurs in the absence of specific antibody. We observed that peptidoglycan fragments from Hi are sufficient to promote neutrophil-mediated phagocytosis of opsonized Sp. Pathways for the recognition of and response to peptidoglycan fragments leading to NF-κB-dependent transcriptional activation and pro-inflammatory responses have been partially characterized [7]. Peptidoglycan fragments containing the minimal structure γ-D-glutamyl-meso-diaminopimelic acid (meso-DAP) found in Gram-negative bacteria, including Hi, act through a cytoplasmic signaling molecule, nucleotide-binding oligomerization domain-1 (Nod1) [8–10]. In the peptidoglycan of most Gram-positive bacteria, including Sp, meso-DAP is

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Abbreviations: CR3, complement receptor 3; DPI, dibenziodolium chloride; Hi, \textit{Haemophilus influenzae}; HKHi, heat-killed \textit{Haemophilus influenzae}; i.p., intraperitoneal; LPS, lipopolysaccharide; MDP, muramyl dipeptide; meso-DAP, γ-D-glutamyl-meso-diaminopimelic acid; Nod1, nucleotide-binding oligomerization domain-1; PEC, peritoneal exudates cell; Sp, \textit{Streptococcus pneumoniae}

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Pathogens are generally studied in the laboratory one species at a time. Most exist, however, in complex environments where they must adapt not only to their host but also to other members of the microbial flora. Using a mouse model of co-colonization, we have shown that one bacterial species (Haemophilus influenzae) can take advantage of the innate immune response of its host to outcompete and eliminate another species (Streptococcus pneumoniae) that resides in the same microenvironment of the upper respiratory tract. The molecular mechanism for this effect involves recognition of a cell wall fragment found on H. influenzae, but not on S. pneumoniae. The response to this immunostimulatory fragment requires Nod1, a host molecule that transmits inflammatory signals in response to specific peptides of the bacterial cell wall. This Nod1-mediated inflammatory stimulation triggers an increase in the ability of a type of white blood cell (neutrophil) to engulf and then kill S. pneumoniae, effectively removing it from its niche on the mucosal surface of the host airway. Our study, therefore, provides a demonstration of the importance of Nod1 in neutrophil-mediated clearance of bacterial infection. In addition, we have described a mechanism for interspecies competition between microbes that occurs through selective stimulation of host innate immune responses.

**Results**

**Killing of Sp Occurs via Opsonophagocytosis**

The increased ability of ex vivo PECs to kill Sp when elicited following intraperitoneal (i.p.) administration of heat-killed Hi (HKHi) allowed us to examine the mechanism whereby one species stimulates the killing of another. When HKHi-stimulated PECs were divided by density gradient centrifugation into mononuclear cell- and neutrophil-containing fractions, only the neutrophil-enriched fraction demonstrated killing of Sp (unpublished data). This result correlated with the absence of killing by HKHi-stimulated PECs when elicited from mice depleted of neutrophils by prior treatment with RB6-8C5, an antibody to murine Ly6G [6,13]. Addition of HKHi correlated with increased neutrophil activation as confirmed by increased expression of the marker Mac-1(complement receptor 3 [CR3], CD11b/CD18) in cells co-expressing Ly6G [6]. Moreover, increased killing of Sp following administration of HKHi was observed with neutrophil-enriched PECs derived from parental but not congenic Mac-1−/− mice (Figure 1). This finding pointed to the requirement of complement-mediated opsonization for neutrophil recognition. When heat-inactivated serum or serum from C3−/− mice was used as a complement source, no killing by HKHi-stimulated neutrophil enriched PECs was seen, confirming the requirement of active complement. Although C3 may be activated by either classical or alternative pathways, killing in the presence of serum from scid mice lacking antibody made it less likely that complement was being activated by the classical pathway [6]. The requirement for the alternative pathway was confirmed by showing a lack of Sp killing when serum from factor B–deficient mice was used as a complement source (Figure 1). Thus, results using PECs indicated that products of Hi stimulate neutrophil-mediated phagocytic killing of Sp opsonized primarily by activation of the alternative pathway of complement.

**Enhancement of Opsonophagocytic Killing Is Independent of TLR2, TLR4, and rPAF**

Products of Hi have previously been shown to signal pro-inflammatory responses through toll-like receptor (TLR) 2 and TLR4, through recognition of its lipopolysaccharide (LPS) and lipoproteins, respectively [14,15]. In addition, platelet-activating factor receptor (rPAF)-mediated signaling has been described for those Hi phase variants expressing the cell surface ligand phosphorylcholine [16]. Opsonophagocytic killing was assessed in neutrophil-enriched PECs derived from TLR2−/− mice. These showed increased killing in response to HKHi and were as active as cells derived from the TLR2-expressing mouse strain (Figure 2). Opsonophagocytic killing was also compared in neutrophil-enriched PECs derived from CH3/OuJ and C57/HeJ mice, which express functional and non-functional TLR4, respectively. TLR4 did not contribute to Sp killing in response to HKHi stimulation. Moreover, HKHi derived from isogenic mutants expressing or not expressing phosphorylcholine stimulated similar levels of Sp killing by neutrophil-enriched PECs (unpublished data) [17]. Together, these results showed that the enhancement of opsonophagocytic killing occurs independently of non-redundant signaling involving known cell surface pattern recognition receptors for Hi, including TLR2, TLR4, and rPAF.

**Enhancement of Opsonophagocytic Killing through Recognition of Peptidoglycan**

This unexpected finding led us to characterize the signal from Hi that enhances the opsonophagocytic killing of Sp. Instead of LPS, purified peptidoglycan from Hi (or Staphylococcus aureus) was less active even when administered at a 10-fold higher dose (Figure 3 and unpublished data). The greater potency of HKHi-stimulated PECs by purified LPS (in doses up to 50 μg/animal) extracted from Hi or Escherichia coli (Figure 3). These findings were also consistent with a signaling pathway other than recognition of Hi components by TLR2, TLR4, or rPAF.

In contrast, purified Hi peptidoglycan at a dose as low as 1 μg/animal was sufficient to stimulate increased killing of Sp by heat-killed Hi-stimulated PECs (activity equivalent to 107 HKHi). Purified peptidoglycan from Sp (or Staphylococcus aureus) was less active even when administered at a 10-fold higher dose (Figure 3 and unpublished data). The greater potency of Hi peptidoglycan correlated with the stimulation of killing by HKHi but not HKSp [6]. This observation indicated that structural differences between cell wall fragments of these species may be important determinants of their peptidoglycan-mediated signaling. To confirm this hypothesis, FK-156, a synthetic muropeptide containing meso-DAP, was tested and showed a level of stimulatory activity equivalent to purified Hi peptidoglycan when administered at an equiva...
Neutrophil-enriched PECs were obtained from mice following i.p. administration of heat-inactivated whole H636 in casein. The effect of HK/Hi on the ability of neutrophil-enriched PECs to kill Sp1121 is shown relative to controls where neutrophil-enriched PECs were elicited by casein administration alone. Survival of Sp1121 was assessed over a 45-min incubation with complement in serum from wild-type mice (WT), C3-deficient mice (C3−/−), or factor B-deficient mice (B−/−) as indicated. Neutrophil-enriched PECs were obtained from Mac1−/− (grey bar) or control C57Bl/6 (black bars) mice. No stimulation of killing was observed in controls using heat-inactivated complement or cells from animals pretreated with monoclonal antibody RB6-8C5 to deplete neutrophils.

Next, we explored the mechanism for increased opsonophagocytic killing of Sp by neutrophil-enriched PECs elicited by casein administration alone. The effect of i.p. administration of heat-inactivated whole H636 in casein on the ability of neutrophil-enriched PECs to kill Sp1121 is shown relative to controls where neutrophil-enriched PECs were elicited by casein administration alone. Values represent ≥ three independent determinations in duplicate ± SD. *p < 0.05 compared to other groups of the same genetic background.

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**Requirement for Nod1 in Enhancement of Opsonophagocytic Killing and Interspecies Competition**

The potency of Hi peptidoglycan, as well as that of FK-156, suggested that stimulation of opsonophagocytic killing involved recognition of Hi components by Nod1. In order to examine this possibility, neutrophil-enriched PECs from Nod1+/− mice were analyzed for their response to HK/Hi and FK-156. As predicted, administration of FK-156 (10 μg/animal) stimulated Sp killing by cells in parental, but not in Nod1+/− mice (Figure 4A). Neutrophil-enriched PECs from Nod1+/− mice also showed a diminished response to HK/Hi, demonstrating that Nod1 accounts for a significant proportion of the signaling generated by innate recognition of this organism.

To further confirm this observation, a meso-DAP-containing peptide, murNAcTriDAP, was synthesized using the Mur enzymes of Gram-negative bacteria [18]. As predicted, its ability to stimulate Sp killing by neutrophil-enriched PECs was equivalent to that of FK-156 and dependent on Nod1 (Figure 4A). In contrast, a synthetic form of the correspond-
results suggested that the recruitment of neutrophils and their migration to mucosal sites with bacteria were not affected by the expression of Nod1 in this model. Additional evidence that Nod1 did not impact neutrophil migration came from comparisons by flow cytometry of PECs elicited by HKHi or HKHi 

Figure 3. The Ability of Bacterial Components to Stimulate Opsonophagocytic Killing of Sp

Neutrophil-enriched PECs were obtained from C57Bl/6 mice pretreated by i.p. administration of casein containing heat-inactivated whole Hifi36 (HKHi, n = 6). E. coli LPS (50 μg/animal, n = 3), Hi LPS (50 μg/animal, n = 4), Sp peptidoglycan (PGN, 10 μg/animal, n = 5), Hi peptidoglycan (PGN, 1 μg or 10 μg/animal, n = 4 and 3, respectively), FK-156 (1.0 μg/animal, n = 6), or MDP (10 μg/animal, n = 3). Survival of Sp1121 is shown relative to controls where neutrophil-enriched PECs were elicited by casein administration alone. Values represent duplicate determinations of the number of independent experiments indicated above for each condition ± SD. *p < 0.01 compared to casein alone control.

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Discussion

Although numerous studies have defined Nod1-mediated effects of bacteria or their cell wall products in vitro, our understanding of its contribution to innate immune responses to bacterial infection in vivo remains limited (reviewed in [7,19]). We demonstrate here that the Nod1 signaling pathway can respond to meso-DAP-containing compounds to increase clearance of Sp from the mucosal surface of the airway. Thus, Nod1 was shown to be important in dictating the outcome of competition between two pathogens that occupy a similar niche in their host [6]. Enhanced killing of Sp required products from another organism, since cell wall fragments from Sp, like most Gram-positive species, do not signal through Nod1. Our findings are relevant to polymicrobial infection and situations in which products from multiple types of organisms are present. This information adds to our previous report, which describes how combinations of microbes and microbial products synergize to enhance inflammatory responses [20]. Mucosal surfaces, in particular, are generally colonized simultaneously with multiple species. The paradigm of one species promoting an innate immune response that affects a competitor may be underappreciated, because most models of infection typically examine responses to individual microbial species. While our model was useful in revealing a role for Nod1 in vivo, it also demonstrates that bacteria that succeed in such environments must have mechanisms to evade its clearance-promoting effects. The specificity for bacterial cell wall components that act through Nod1 suggests a mechanism whereby many Gram-positive pathogens that lack meso-DAP may avoid signaling events that lead to neutrophil-mediated killing. Likewise, the density of colonizing Hi during co-infection was not affected by Nod1 signaling, in contrast to clearance of Sp during co-colonization. This suggests that Hi may be resistant to the response induced by its meso-DAP-containing peptidoglycan, and also to the enhancement of opsonophagocytic killing by neutrophils seen against Sp. In addition to the synthesis of stem peptides without meso-DAP, there may be multiple mechanisms to evade peptidoglycan recognition and stimulation of immune signaling through Nod1 [21]. For example, it has recently been reported that modification of the 2-carboxylic acid group of iso-glutamic acid, the residue proximal to meso-DAP, to an amide diminishes signaling through Nod1 and may be a mechanism for immune invasion by some pathogens [22]. Both Sp and Hi are considered extracellular pathogens, which are unable to effectively access intracellular pathways [23]. In the case of epithelial cells, pore-forming toxins or delivery via the type IV pilus have been shown to be necessary for peptidoglycan to gain access to the cytoplasm [24,25]. Moreover, Nod1-deficient mice were shown to be more susceptible to infection by Helicobacter pylori expressing the cag pathogenity island type IV secretion apparatus than were wild-type mice [25]. Our observation in this report that peptidoglycan fragments alone are sufficient to induce Nod1-dependent effects shows that access to these cytoplasmic pathways may not be similarly limited for professional phagocytes. In killing assays, however, bacteria or peptidoglycan fragments were delivered in vivo and their activity tested ex vivo. Thus, we cannot confirm whether the effect of injected compounds or bacterial products on neutrophil function was direct. Attempts to treat neutrophils in vitro with immunostimulatory fragments that are active when provided in vivo were not sufficient to elicit a direct effect in killing assays. It is unlikely that this is due to a lack of Nod1
expression in these cells, because in contrast to other members of the Nod protein family, Nod1 expression is ubiquitous [7]. It remains possible that Nod1-mediated signaling requires other cell types, such as epithelial cells, and that its effects on neutrophil function are indirect.

A further consideration is that neutrophils have cell wall–degrading enzymes, such as lysozyme, that may generate more biologically active peptidoglycan fragments. This could account for the effects of purified peptidoglycan in our study, which contrasts with prior reports where only synthetic products are active. Thus, both the processing of peptidoglycan and the ability of cell wall fragments to access the cytoplasm may be important factors for signaling events involving neutrophils. In this regard, it has been suggested that Sp and other Gram-positive pathogens synthesize modified peptidoglycan that is resistant to lysozyme [21,26,27]. Thus, a number of adaptations may contribute to minimizing Nod-mediated signaling by Gram-positive bacteria despite their greater quantity of peptidoglycan per cell.

Our study demonstrates that the resistance of Sp to killing by neutrophils (Figure 4B) can be overcome by a specific immune signaling pathway. Findings in this study with microbial products and synthetic meso-DAP-containing peptidoglycan fragments add to a prior report that systemic administration of FK-156 enhanced host resistance to various microbial infections [28]. Bacterial killing in our system required opsonization, which for Sp strain Sp1121 occurred through activation of the alternative pathway of complement, followed by phagocytosis by activated, Mac-1 (CR3, CD11b/CD18)-expressing neutrophils. One of the ligands of Mac-1, or CR3, is iC3b [29]. It remains unclear how Nod1-mediated signaling enhances Mac-1-dependent opsonophagocytic killing of complement-opsonized Sp. It has been suggested that Nod1 transduces signals that can stimulate chemokine production and neutrophil recruitment [30]. We did not observe, however, a Nod1-related effect on the increase in MIP-2 levels or influx of neutrophils into either the peritoneal cavity or the nasal spaces in response to bacteria. Likewise, no effect of Nod1 on the uptake of bacteria or generation of an oxidative burst was detected. Rather, killing of Sp in our model resulted from stimulation of non-oxidative activity of neutrophils. Reduced killing in the presence of inhibitors of actin polymerization and rearrangement, and the requirement for complement, argue against Nod1-mediated enhancement of previously described mechanisms for extracellular killing of Sp by neutrophils [31]. We are currently characterizing this oxidative burst–independent anti-pneumococcal effect of neutrophils and the

![Figure 4. The Ability of Synthetic Peptidoglycan Fragments to Stimulate Opsonophagocytic Killing of Sp](image-url)

**A** Neutrophil-enriched PECs were obtained from C57Bl/6 (black bars) or congenic Nod1−/− (grey bars) mice pretreated by i.p. administration of casein containing heat-inactivated whole Hi636 (HKHi), murNAcTriDAP (DAP, 10 μg/animal), or its synthetic analog, FK-156 (10 μg/animal). Survival of Sp1121 is shown relative to controls where neutrophil-enriched PECs were elicited by casein administration alone. Values represent ≥ three independent determinations in duplicate ± SD. *p < 0.01 compared to the same treatment in parental mice.

**B** Neutrophil-enriched PECs were obtained from C57Bl/6 mice pretreated by i.p. administration without casein of heat-inactivated whole Hi636 (HKHi), murNAcTriDAP (DAP, 10 μg/animal), or murNAcTRILys (LYS, 10 μg/animal). Values represent ≥ three independent determinations in duplicate ± SD. *p < 0.01 compared to other groups.

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![Figure 5. Effect of Nod1 on Competition between Species during Co-Colonization](image-url)

The density of Sp1121 (Sp) in upper respiratory tract lavage was determined at 24 h post-intranasal inoculation with Sp alone or together with Hi636 (Hi) in Nod1−/− or parental C57Bl/6 mice (WT). Box-and-whiskers plot indicates high and low values, median and interquartile ranges; n ≥ 10 mice from three independent experiments in each group. Co-inoculated species shown in parentheses. The lower limit of detection for bacteria in lavage culture was 10^2 CFU/ml (indicated by a dashed line).

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contribution of Nod1 to stimulation of this biological activity.

Findings in this study also show a limited role of other signaling pathways in clearance of Sp from the mucosal surface of the murine airway. Sp has previously been shown to activate cellular NF-κB-dependent immune responses through Nod2. However, the effect of fragments acting through Nod2, including MDP, purified Hi or Sp peptidoglycan, and live or killed Hi or Sp, was minor in comparison to those acting through Nod1 [6,32]. Moreover, the Hi-induced increase in Sp killing by neutrophil-enriched PECs was not influenced by the pathogen-associated molecular pattern receptors, TLR2 or TLR4, in a non-redundant manner. Thus, our study provides an example where the predominant signaling response of the innate immune system to a bacterial challenge appears to be through Nod1.

Materials and Methods

Bacterial strains and culture conditions. Hi and Sp strains were grown as previously described [33]. Strains used in vivo were selected because of their ability to colonize efficiently the murine nasal mucosa and included Hi 636 (a type b capsule-expressing, spontaneously streptomycin-resistant mutant of Hi strain Eagan), and Sp 1121 (a type 23F capsule-expressing Sp isolate from the human nasopharynx [34]). Genetically modified Hi mutants of strain Eagan were also used. An in vitro assay was used to evaluate the ability of strains to induce chemokine production, neutrophil recruitment, and phagocytosis in murine nasal mucosa.

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that constitutively express or lack phosphorylcholine on its LPS were previously described [17].

**Mouse strains.** Six-week-old mice used in the study were housed in accordance with Institutional Animal Care and Use Committee protocols. Mouse strains included C57Bl/6j and congenic Nod1−/− (Millipore, Billerica, MA; http://www.millipore.com), B6.Cg-Itgam−/− (Jackson Laboratories, Bar Harbor, ME; http://www.jax.org/), and BALB/cByJ (Jackson Laboratories, Bar Harbor, ME; http://www.jax.org/). Neutrophil or monocytic cell-enriched fractions were again 2 h prior to cell harvest by i.p. administration of 10% casein in animal with PBS containing 20 mM EDTA) of mice treated 24 h and ml neomycin to select for Sp,

Mg assayed by combining 10 2 PBS-washed, mid-log phase bacteria (in 10 l), and Hank's buffer with Ca

8 heat-inactivated by treatment at 65

lg/ml of trypsin (Worthington Biochemical, http://www.worthington-biochem.com/) in the presence of 10 mM CaCl2. To stop the reaction, 10 mM EGTA was added and the preparation was boiled in 5% SDS for 30 min. After extensive washing, Hi peptidegycan was lyophilized and resuspended at 5 mg/ml in endotoxin-free water.

**Preparation of peptidoglycan fragments.** N-acetylglucosyl-muramyl-L-alanyl-γ-D-glutamyl-meso-2,6-diaminopimelic acid (murNAcTRI DAP), and N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-lysine (murNAcTRILYS) were prepared as described previously [18,45,46]. Briefly, recombinant pseudomonas aeruginosa (Pa) MurA, MurB, MurC, and MurD were used to synthesize uridine 5′diphosphoryl-N-acetylglucosyl-l-alanyl-γ-D-glutamic acid (UDP-murNAcDi); additionally, Ps MurE was used to synthesize uridine 5′diphosphoryl-N-acetylmuramyl-l-alanyl-γ-D-glutamyl-L-lysine (UDP-murNAcTRILYS). Electrospray ionization mass spectrometry (negative ion) was used to confirm the molecular weight of synthesized compounds. Purity was assessed by analytical anion exchange chromatography using a GE Healthcare Mono Q HR5/5 column (http://www.gelifesciences.com) and by continuous spectrophotometric enzyme assay with MurE for UDP-murNAcDi, and MurF for UDP-murNAcTRILYS. N-acetylglucosaminyl-peptides were produced by mild acid hydrolysis (1 M HCl, 1 h) of the corresponding uridine 5′diphosphoryl-N-acetylmuramyl-peptides. Complete hydrolysis was confirmed by continuous spectrophotometric enzyme assay with MurE for murNAcDi (MDP), and MurF for murNAcTRILYS. Peptides were analysed by electrospray ionisation mass spectrometry (positive ion) and by a GE Healthcare Mono Q HR5/5 column (http://www.gelifesciences.com) and by continuous spectrophotometric enzyme assay. Corresponding concentrations of UDP, UMP, and P1 were estimated by continuous spectrophotometric enzyme assay by comparison to controls with inactivated peptide at 65 °C for 30 min and shown to be non-viable.

**Phagocytic killing assays.** Neutrophil-enriched PECs were counted by trypsin blue staining and adjusted to a density of 7 × 106 cells/ml. Killing during a 15 min incubation at 37 °C with rotation was assessed by combining 105 PBS-washed, mid-log phase bacteria (in 10 μl) with complement source (in 20 μl), 105 mouse phagocytes (in 40 μl), and Hank’s buffer with Ca2+ and Mg2+ (GIBCO) plus 0.1% gelatin (130 μl). Earlier time points and fewer effector to target cells were assessed in pilot experiments to reduce in loss. The complement source consisted of fresh mouse serum from C57Bl/6j mice unless indicated otherwise. After stopping the reaction by incubation at 4 °C, viable counts were determined in serial dilutions. Percent killing was determined relative to the same experimental conditions with no co-administered casein. To inhibit phagocytosis, neutrophils were preincubated with cytochalasin D (0.9 μM, Sigma) for 15 min at 37 °C. Intracellular pneumococci were quantified using viable counts following the addition of gentamicin sulfate (final concentration 300 μg/ml). After a 20-min incubation at 37 °C, the antibiotic was removed by serial washing prior to plating for viable counts.

**Isolation of peptidoglycan and other bacterial components.** Hi LPS was purified by hot-phenol extraction from strain Eagan as previously described [41]. E. coli LPS and Staphylococcus aureus peptidoglycan were purchased from Sigma.

**Preparation of peptidoglycan from Hi was modified from a previously described protocol [42]. Briefly, strain H636 was grown overnight in sBHI, pelleted at 6,000g at 4 °C, and washed with Tris-buffered saline (TBS). The pellet was resuspended in 5 ml of cold g/ml of trypsin (Worthington Biochemical, http://www.worthington-biochem.com/) in the presence of 10 µM CaCl2 and 10 mM EDTA and the peptidoglycan preparation was boiled in 5% SDS for 30 min. After extensive washing, Hi peptidegycan was lyophilized and resuspended at 5 mg/ml in endotoxin-free water.

**Preparation of peptidoglycan fragments.** N-acetylglucosyl-muramyl-L-alanyl-γ-D-glutamyl-meso-2,6-diaminopimelic acid (murNAcTRI DAP), N-acetylmuramyl-L-alanyl-γ-D-glutamic acid (murNAcDi), and N-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-lysine (murNAcTRILYS) were prepared as described previously [18,45,46]. Briefly, recombinant pseudomonas aeruginosa (Pa) MurA, MurB, MurC, and MurD were used to synthesize uridine 5′diphosphoryl-N-acetylglucosyl-l-alanyl-γ-D-glutamic acid (UDP-murNAcDi); additionally, Ps MurE was used to synthesize uridine 5′diphosphoryl-N-acetylmuramyl-l-alanyl-γ-D-glutamyl-L-lysine (UDP-murNAcTRILYS). Electrospray ionization mass spectrometry (negative ion) was used to confirm the molecular weight of synthesized compounds. Purity was assessed by analytical anion exchange chromatography using a GE Healthcare Mono Q HR5/5 column (http://www.gelifesciences.com) and by continuous spectrophotometric enzyme assay with MurE for UDP-murNAcDi, and MurF for UDP-murNAcTRILYS. N-acetylglucosaminyl-peptides were produced by mild acid hydrolysis (1 M HCl, 1 h) of the corresponding uridine 5′diphosphoryl-N-acetylmuramyl-peptides. Complete hydrolysis was confirmed by continuous spectrophotometric enzyme assay with MurE for murNAcDi (MDP), and MurF for murNAcTRILYS. Peptides were analysed by electrospray ionisation mass spectrometry (positive ion) and by a GE Healthcare Mono Q HR5/5 column (http://www.gelifesciences.com) and by continuous spectrophotometric enzyme assay. Corresponding concentrations of UDP, UMP, and P1 were estimated by continuous spectrophotometric enzyme assay by comparison to controls with inactivated peptide at 65 °C for 30 min and shown to be non-viable.

**Histology and immunofluorescence.** At 24 h post-inoculation, the animal was sacrificed and decapitated, and the head was fixed for 48 h in 4% paraformaldehyde in PBS. The head was then decalcified by 0.1 M EDTA (pH 7.0) at 4 °C over 1 mo before freezing in Tissue-Tek O.C.T. embedding medium (Miles, Elkhart, Indiana, United States) in a Tissue-Tek Cryomold. Then, 5-μm thick sections were cut, air dried, and stored at −80 °C. Frozen-embedded tissue sections were stained with hematoxylin and eosin (H&E) following a 10-min fixation step in 10% neutral buffered formalin.
(NBF). Sections were then dehydrated in alcohol, cleared in xylene, and mounted in cytoskeletal (Richard-Allan Scientific, http://www.rallansci.com). Immunofluorescent staining on frozen tissue was performed and visualized as previously described [47]. Neutrophil-like cells were stained using rat anti-mouse Ly6G mAb (BD Biosciences) followed by anti-rat Ig secondary antibody [6]. To detect Sp1121, sections were incubated with antisera to Sp type 239 (Statens Serum Institut, http://www.sssi.dk/) followed by anti-rabbit Ig secondary antibody. To detect H636, sections were incubated with antisera to Hi type b (DIFCO Laboratories, http://www.bdlbiosciences.com/).

**Measurement of MIP-2 concentration.** Upper respiratory tract lavage fluid was assayed for the concentration of macrophage inhibitory protein (MIP-2) by ELISA in duplicate according to the manufacturer's instructions (Pharmingen, http://www.bdbiosciences.com/).

**Statistical analysis.** Statistical comparisons of colonization among groups were made by the Kruskal–Wallis test with Dunn’s post-test (GraphPad Prism 4; GraphPad Software, http://www.graphpad.com/).

**Results**

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