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Mucosal Clearance of Capsule-Expressing Bacteria Requires Both TLR and Nucleotide-Binding Oligomerization Domain 1 Signaling

Tracey A. Zola,* Elena S. Lysenko,* and Jeffrey N. Weiser2g†

Expression of capsular polysaccharide by bacterial pathogens is associated with increased resistance to host clearance mechanisms, in particular by evading opsonization and uptake by professional phagocytes. The potential for rapid progression of disease caused by encapsulated bacteria points to the importance of innate immunity at the mucosal surface where infection is initiated. Using a murine model of nasopharyngeal colonization, host immune components that contribute to the mucosal clearance of capsule-expressing bacteria were investigated. Clearance of encapsulated Haemophilus influenzae (Hi) required both TLR and nucleotide-binding oligomerization domain (NOD) signaling pathways, whereas individual deficiencies in each of these signaling cascades did not affect clearance of nonencapsulated strains. Moreover, clearance of Hi-expressing capsular polysaccharide required the recruitment of neutrophils to the site of infection, and ex vivo phagocytic bacterial killing required expression of the NOD1 signaling pathway. Conversely, redundancies within these innate immune pathways of non-neutrophil cells were sufficient to promote mucosal clearance of nonencapsulated Hi. Our findings reveal a role for NOD1 in protection from encapsulated pathogens. In addition, this study provides an example of a microbial virulence determinant that alters the requirements for host signaling to provide effective protection. The Journal of Immunology, 2008, 181: 7909–7916.

Any major bacterial pathogens express a thick coat of capsular polysaccharide on their surface. Their capsules render them more resistant to host clearance mechanisms, particularly those involving opsonization by complement and/or Ab followed by uptake by professional phagocytes (1, 2). These attributes enhance the ability of extracellular bacteria to survive within the bloodstream and explain why encapsulated pathogens are among the most common agents causing severe invasive infections. However, many encapsulated organisms reside on mucosal surfaces where they exist primarily in a commensal relationship with their host. In the human nasopharynx, clinically important examples of these encapsulated organisms include Haemophilus influenzae (Hi) type b (Hib), Streptococcus pneumoniae, and Neisseria meningitidis. Nasal carriage of these species is generally transient but a prerequisite for the development of disease (3, 4). Invasive infection is a relatively rare outcome but it may be both overwhelming and expedient, occurring within days from the establishment of colonization. Although capsular polysaccharides are often immunodominant Ags, adaptive immune responses may develop too slowly to confer protection during the initial period following bacterial acquisition. This suggests that innate immunity must be crucial for protection against encapsulated mucosal pathogens.

The cellular components of innate immunity use pattern recognition molecules (PRMs) that recognize microbial expressed pathogen-associated molecular patterns. Bacterial pathogen-associated molecular patterns trigger responses through interactions with PRMs including TLRs and nucleotide oligomerization domain (NOD) proteins (for review see Refs. 5, 6). In particular, TLR4 and TLR2 signaling pathways represent key surface regulators of innate immune responses to extracellular bacteria through the recognition of bacterial LPS and lipid-modified components, respectively. Additionally, the cytoplasmic NOD proteins are involved in innate immune signaling events through recognition of specific peptidoglycan motifs. NOD1, for instance, signals in response to a diaminopimelic acid-containing peptid moiety (7, 8). After recognition of bacterial components by these PRMs, distinct intracellular signaling events are elicited, resulting in the eventual activation of NFκB and initiation and/or modulation of innate and adaptive immune responses. Cooperation and redundancy among these innate immune detectors is of critical importance to regulating and shaping antimicrobial immunity.

Capsule-mediated modulation of bacterial interactions with PRMs has been suggested for a variety of bacterial species including S. pneumoniae (9), Salmonella enterica serotype typhi (10), and Streptococcus suis (11). Encapsulated organisms transiently residing within the nasopharynx are capable of causing rapid and serious infections as a result of overcoming host defense mechanisms. Therefore, we set forth to investigate the host immune components required for mucosal clearance of bacteria in vivo. In this study, Hi was used as a model pathogen since both capsule-expressing and nonexpressing (or nontypeable) forms naturally exist and are capable of initiating infection, and a role of capsule in facilitating bacterial survival within the host has been well established (12, 13). We found that in the absence of individual PRMs,
clearance of nonencapsulated bacteria remains effective. Conversely, the absence of TLR2, TLR4, or NOD1 signaling pathways, and depletion of neutrophil-like cells, attenuates clearance of Hi-expressing capsular polysaccharide. Our findings highlight the differing requirements for protection by innate immune mechanisms for encapsulated and nonencapsulated mucosal pathogens.

Materials and Methods

Mouse strains

The following strains of mice were obtained from Jackson ImmunoResearch Laboratories: C57BL6 (wild type (WT)), B6.129-S2-Igh-6tm1Cgn/J (µMT), B6.CB17-Prkdcscid/SCid, and C57BL10ScNJ (TLR4−/−). µMT mice contain a targeted deletion in the H chain locus of C57BL6 Ig M (IgM) and do not produce mature B cells or Ab (14). SCID mice contain a spontaneous mutation in a gene encoding the catalytic subunit of DNA-activated protein kinase, resulting in the absence of B and T cells (15). TLR4-deficient mice contain a deletion of the tlr4 gene resulting in defective response to LPS stimulation (16).

Polymeric (pIgR-deficient mice (C57BL6-pIgR−/−) were purchased from Taconic and contain a targeted deletion of the pIgR locus resulting in animals lacking secretory IgA (17). NOD1-deficient mice were obtained from Millennium Pharmaceuticals and contain a targeted mutation of CARD4. The genotype was confirmed as described previously (18).

Studies were conducted in accordance with the guidelines of the University of Pennsylvania, and all mice were housed in accordance with Institutional Animal Care and Use Committee protocols. Water and a standard rodent diet were provided ad libitum. Mice included both males and females inoculated at the age of 5 to 8 wk unless otherwise specified.

Bacterial strains and culture conditions

Hi strains were grown in Brain heart infusion broth (BD Biosciences) supplemented with 2% Filde’s Enrichment (Remel) and 20 µg/ml B-NAD (Sigma-Aldrich). Strains were previously described and included: H636, Eagan type b capsule (Hib); H648, a spontaneous b mutant of Eagan lacking both copies of the cap locus (21); H631, NTHI strain Tn106.F2 (22); H632, NTHI strain SR7332 (23); and H630, NTHI strain 86.02SNP (24). Strains H631 and H632 were used because they had been previously shown to persist in the murine airway. H680 was chosen because of the availability of its entire genomic sequence. All strains used in experiments were spontaneously streptococcytin-resistant mutants and animal passaged.

Mouse model of nasopharyngeal colonization

Mice were inoculated intranasally with 10 µl containing 10−20 CFU of PBS-washed, mid-log phase Hi. The animal was sacrificed at the appropriate time point, the trachea cannulated, and 200 µl of PBS instilled (500 µl of PBS was used for cytokinin preparation). Lavage fluid was collected from the nares for determination of viable counts of bacteria in serial dilutions plated on selective medium containing streptomycin (100 µg/ml) to inhibit the growth of contaminants. The lower limit of detection for bacterial colonies was 10 CFU/ml.

Histology and immunofluorescence

At the time indicated post inoculation, the animal was sacrificed and decapitated. The head was fixed for 2 days in 4% paraformaldehyde and decalcified in 0.12 M EDTA (pH 7.0) for 30 days. The heads were then frozen in Tissue-Tek OCT embedding medium (Electron Microscopy Sciences) and 5-µm-thick sections were cut and either stained with H&E or stored at −80°C. For immunofluorescence, sections were postfixed in 1:1 methanol/acetic acid at −20°C for 10 min followed by washing with PBS. Nonspecific binding was inhibited by incubating for 10 min with protein-blocking reagent (Coulter-Immunotech). Sections were then incubated for 1 h at room temperature with primary Abs including polyclonal rabbit H. influenzae antiserum type b (BD Biosciences) to detect bacteria (1/400) and rat anti-mouse Ly6G to detect neutrophils (1/200).

After PBS washing, secondary Abs including Cy3-conjugated donkey anti-rabbit IgG (1/400) to detect bacteria and Cy2-conjugated donkey anti-rat IgG (1/400) to detect neutrophils (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After washing with PBS followed by dH2O, sections were counterstained with 4’6-diamidino-2-phenylindole (Molecular Probes, Invitrogen) diluted 1/10,000 in dH2O. All imaging was performed using a Nikon Eclipse E600 microscope equipped with a high-resolution charge-coupled device digital camera (CoolSNAP). All image analysis was conducted using IPLAB (Scanalytics).

Isolation and characterization of murine phagocytes

Peritoneal exudate cells (PECs) were isolated from 10-wk-old C57BL6 mice as previously described (28). In brief, phagocytes were obtained by lavage of the peritoneal cavity (8 ml/animal with PBS containing 0.02 M EDTA) of mice treated 1 day and again 2 h before cell harvest by i.p. administration of 10% casein in PBS (1 ml per dose). In some experiments, where indicated, cells collected from the peritoneal cavity were enriched for neutrophils using separation in a Ficoll density gradient centrifugation using Nunc-Poly Resolving Medium according to the manufacturer’s instructions (MP Biomedicals). This neutrophil-enriched fraction was collected and washed with 5 ml of Hank’s buffer without Ca2+ or Mg2+ (Invitrogen) plus 0.1% gelatin. This population of cells was characterized previously using flow cytometric analysis for staining of granulocytes with anti-mouse Ly-6G mAb and CD11b (BD Biosciences) (18). Total PECs were collected from the peritoneal cavity of casein-treated C57BL6, TLR2−/−, and NOD1−−/− mice and washed with 5 ml of Hank’s buffer without Ca2+ or Mg2+ (Invitrogen) plus 0.1% gelatin. Flow cytometric analysis was performed on PECs to characterize the relative proportions of activated neutrophils (Ly-6G+, CD11b+) and macrophages (F4/80−, CD11b+). Some mice, where indicated, were pretreated with RB6-8C5 mAb (as described above) before initial casein injection and isolation of total PECs.

Phagocytic killing assays

Neutrophil-enriched or total PECs were counted by trypan blue staining and adjusted to a density of 7 × 106 cells/ml. Killing during a 45-min incubation at 37°C with rotation was assessed by combining 10 µl of 106 PBS-washed, mid-phase bacteria preopsonized with 40 µl of a complement source, and 40 µl containing 106 murine neutrophil-enriched or total PECs, and 130 µl Hank’s buffer with Ca2+ and Mg2+ (Invitrogen) plus 1% gelatin. The complement source consisted of fresh serum from either unfected C57BL6/6J (WT) or µMT (Ab-deficient) mice. In some cultures, complement was inactivated by heating to 56°C for 30 min before mixing with bacteria. A total of 10,000 cells was collected for each sample, and groups were compared using FlowJo software (Tree Star).
Innate immunity limits Hib colonization. The density of Hi-expressing type b capsular polysaccharide in the upper respiratory tract was determined to be 300 CFU/ml of H9262, which is consistent with previous findings (29). A primer set was designed to target two T4SS genes exhibiting homology to traB and pilT, and to target paraA, a putative replication region, as previously described (29). A primer set to betT (positive control) was also designed (Forward: 5′-GCGCTGACCTGTTCTGATATAACCCATT-3′; Reverse: 5′-CCCAAGATTTGAAAGATACACAATGGTTTGATTTAAAA-3′). Portions of paraA, traB, pilT, and betT were amplified with Tag Polymerase (Invitrogen) according to manufacturer’s instructions. After an initial denaturation step of 2 min at 94°C, DNA was amplified for 30 cycles, with each cycle consisting of 45 s at 94°C, 1 min at 48°C, and 1.5 min at 72°C, followed by a final extension step of 7 min at 72°C.

**Genomic DNA isolation and PCR analysis of T4SS-specific genes**

Hi strains were grown to mid-log phase, and chromosomal DNA was isolated by Wizard Genomic DNA Purification Kit according to the manufacturer’s protocol for Gram-negative bacteria (Promega). For detection of the genomic island that includes the T4SS expressed by some Hi strains, primer sets were designed to target two T4SS genes exhibiting homology to traB and pilT, and to target paraA, a putative replication region, as previously described (29). A primer set to betT (positive control) was also designed (Forward: 5′-GCGCTGACCTGTTCTGATATAACCCATT-3′; Reverse: 5′-CCCAAGATTTGAAAGATACACAATGGTTTGATTTAAAA-3′). Portions of paraA, traB, pilT, and betT were amplified with Tag Polymerase (Invitrogen) according to manufacturer’s instructions. After an initial denaturation step of 2 min at 94°C, DNA was amplified for 30 cycles, with each cycle consisting of 45 s at 94°C, 1 min at 48°C, and 1.5 min at 72°C, followed by a final extension step of 7 min at 72°C.

**Statistical analysis**

Statistical comparisons of colonization among groups were made by the Mann-Whitney U test (GraphPad Software) unless otherwise specified.

**Results**

**Clearance of Hib colonization requires innate immunity**

To investigate host and bacterial factors involved in mucosal clearance of Hi, we used a murine model of nasopharyngeal colonization. We identified an encapsulated Hib exhibiting detectable levels of colonization of C57BL6 mice by isolating bacteria from the nasal lavage fluid of infected mice. Colonization levels were examined at 1, 3, and 14 days p.i. revealing variable colonization levels at day 1, a low level of Hib colonization at day 3, and no detectable bacteria by 14 days (Fig. 1 and data not shown). Since limited colonization of Hib was observed at day 3 p.i., indicating effective clearance, this time point was used in additional experiments to evaluate the role of host factors in this process.

Mucosal clearance of Hib at day 3 p.i., before the predicted onset of adaptive immune responses, suggests that innate immunity is essential. Therefore, we set forth to directly rule out a role of adaptive immune responses in clearance by inoculating SCID mice with Hib. The majority of animals were able to clear colonization by day 3 p.i. (Fig. 1). As a supplementary method of investigating the role of Ab in Hib clearance, plgR-deficient mice were tested. These mice, who do not transport Ab to the mucosal surface, did not exhibit attenuated clearance of Hib when compared with the WT mice (Fig. 1). Cumulatively, these data suggest that innate rather than adaptive immunity is essential for mucosal clearance of Hi. Therefore, we examined the involvement of specific innate immune components, including inflammatory cells, complement, and PRMs in the clearance of colonization.

To assess whether mucosal clearance of Hib is neutrophil dependent, mice were treated with RB6-8SC5, a rat mAb recognizing murine Ly6G, before intranasal challenge. This treatment has been shown to deplete neutrophils from peripheral blood (26) and prevent their recruitment to the nasopharynx in colonized mice (30). Mice treated with RB6-8SC5 had an increased density of Hib colonization at day 3 p.i. as compared with WT (p = 0.0005), indicating attenuated clearance (Fig. 1). To determine the role of complement in clearance, mice were treated with cobra venom factor before bacterial challenge to induce hypocomplementemia. Although clearance was not attenuated in many treated mice, some mice exhibited high levels of Hib colonization (Fig. 1), suggesting that clearance may be enhanced by complement deposition.

To investigate the role of a broad range of innate immune signaling pathways in mucosal clearance, colonization of MyD88-deficient mice by Hib was analyzed. MyD88 is involved in transmittting activation signals from most TLRs and IL-1R (31). MyD88-deficient mice exhibited an increased level of Hib colonization as compared with WT mice (Fig. 1) (p < 0.0001), demonstrating attenuated bacterial clearance. Cumulatively, these data reveal a role for innate immunity, specifically neutrophils and MyD88 signaling, in mucosal clearance of Hib.

**Hib colonization induces an acute inflammatory response**

Since innate immune components are essential to the effective clearance of Hib, we characterized the inflammatory response induced by colonization by using histological examination of colonized nasal tissues. This approach demonstrated that Hib induced an influx of inflammatory cells into lateral nasal spaces by 3 h p.i. with a maximal response by 24 h p.i. (Fig. 2A). Immunofluorescent staining of frozen tissue from 24 h-infected mice demonstrated that the infiltrate contained dense clusters of Ly6G-staining neutrophils associated with the bacteria (Fig. 2B). To determine whether macrophages were also recruited to the site of infection, cytospins of nasal lavage fluid were prepared and monocyte/macrophage cells were enumerated. However, an average of <10 monocyte/macrophage cells were identified within the lavage fluid of each animal (data not shown).

**Neutrophils contribute to killing of Hib and clearance of colonization**

To determine whether murine neutrophils, which were recruited to the nasal spaces in response to Hib colonization, are involved in its elimination, neutrophils enriched from elicited PECs were analyzed in ex vivo killing assays. Neutrophil-enriched PECs were incubated with preopsonized Hib. Hib-killing was observed when bacteria were preopsonized with sera isolated from uninfected C57BL6 mice (Fig. 2C). Killing was independent of the presence of specific Ab since serum isolated from Ab-deficient mice (μMT) resulted in equivalent levels of Hib killing. Heat-inactivation of sera before opsonization resulted in minimal neutrophil-enriched PEC-mediated Hib killing, indicating complement-dependence. Cumulatively, these data reveal the recruitment of neutrophils to the site of Hib colonization (Fig. 2, A and B) that is essential for mucosal clearance (Fig. 1). Neutrophils were associated with the bacteria (Fig. 2B) and capable of killing Hib ex vivo (Fig. 2C).
Moreover, the neutrophilic infiltrate is no longer present 3 days p.i. (Fig. 2A), corresponding with the low level of residual bacterial colonization appreciated at this time point (Fig. 1).

**Expression of TLR2, NOD1, and non-neutrophil TLR4 are essential for clearance of Hib**

Since MyD88-deficient mice exhibited attenuated bacterial clearance (Fig. 1), we set forth to further define which individual MyD88-dependent signaling pathways may be involved in the mucosal clearance of Hib. This was accomplished by examining the role of the specific pattern recognition receptors, TLR4 and TLR2, by use of TLR4- and TLR2-deficient mice. Additionally, the role of the pattern recognition molecule NOD1 was also investigated using NOD1-deficient mice. Mice deficient in TLR2, TLR4, or NOD1 expression showed increased levels of bacterial colonization as compared with WT mice at day 3 p.i. indicating a role for each receptor pathway in mucosal clearance of Hib (Fig. 3A) (p < 0.0001; p = 0.0002; p = 0.0001, respectively). Moreover, unlike WT mice, colonization of these immune-deficient mice persisted for at least 14 days p.i. (Fig. 3B). Contrary to what was observed for TLR2 and NOD1-deficient mice, the density of colonizing Hib in TLR4−/− mice significantly increased between days 3 and 14 p.i. (p = 0.002; Fig. 3B), suggesting that the mucosal clearance of these mice remained attenuated even after the expected time frame for the initiation of adaptive immune defenses. Cumulatively, these data demonstrate that the absence of expression of each individual signaling pathway was sufficient to result in increased and more persistent Hib colonization.

Interestingly, when TLR4−/− mice were depleted of neutrophils before bacterial inoculation, the observed colonization levels of Hib were increased as compared with control TLR4−/− mice (p = 0.0002; Fig. 3A). These data reveal an additive effect of non-neutrophil TLR4 expression and neutrophil influx on the mucosal clearance of Hib. Conversely, depletion of neutrophils from TLR2 and NOD1-deficient mice before Hib inoculation did not result in an additional increase in colonization levels as compared with respective knockout mice without neutrophil depletion (Fig. 3A).

Therefore, Hib stimulation of TLR2 and/or NOD1 may act on the same pathway as neutrophils to induce mucosal clearance. In contrast, the greater effect of TLR2 or NOD1 deficiency compared with neutrophil depletion may indicate that these pattern recognition proteins also function on non-neutrophil cell populations.

To determine whether neutrophil-recruitment to the site of infection is observed in Hib-colonized TLR2-deficient and...
To investigate phagocytic killing without limiting analysis to a specific cell type, elicited PECs (without neutrophil enrichment) were isolated from the murine peritoneal cavity of WT, TLR2−/−, and NOD1−/− mice. Flow cytometric analysis of these exudates indicated that the majority of CD45+ cells were activated neutrophils (Ly6G+ and CD11b+) and macrophages (F4/80+ and CD11b+) (data not shown). Incubation of WT phagocytes with preopsonized Hib resulted in complement-dependent bacterial killing. Pretreatment of mice with RB6-8C5 before isolation of total PECs to deplete Ly6G+ cells resulted in no PEC-mediated bacterial killing (data not shown). This provides further evidence that Hib killing by total PECs is mediated by activated neutrophils; either by direct phagocytic activity and/or signaling involved in the recruitment of other phagocytic cells. Phagocytes isolated from TLR2-deficient mice exhibited less killing of Hib as compared with WT (p = 0.04; Fig. 3D), demonstrating that expression of this pathway aids in effective phagocytic killing of encapsulated Hi. Moreover, no bacterial killing was observed with phagocytes isolated from NOD1-deficient mice (Fig. 3E), indicating that expression of this pathway is required for killing of the encapsulated Hi strain by phagocytic cells.

**Limited colonization by noncapsulated Hi**

To investigate the role of polysaccharide capsule expression in mucosal clearance of Hi, we first attempted to identify nonencapsulated strains (NTHi) that exhibit detectable levels of colonization. To investigate the role of polysaccharide capsule expression in mucosal clearance of Hi, we first attempted to identify nonencapsulated strains (NTHi) that exhibit detectable levels of colonization (Fig. 4). At day 3 p.i., low levels of one nonencapsulated strain (H631) were isolated from the nasal lavage fluid of infected mice. These levels were similar to that observed for Hib (H636) and indicate stable colonization of these strains. No detectable colonization of the noncapsulated strains, H632 and H680, was observed. Therefore the nonencapsulated strain, H631, was used in additional experiments to determine whether expression of polysaccharide capsule alters the requirement of various innate immune signaling pathways to promote effective mucosal clearance.

Phagocytes were incubated with H636 preopsonized with sera from uninfected C57BL6 mice for 45 min, and survival was assessed compared with non-phagocyte control groups. No stimulation of killing was observed in controls using heat-inactivated sera. Values are based on three or four independent determinations in duplicate ± SD. Statistical differences were determined using the Paired t test; *, p < 0.05; **, p < 0.01.
nonencapsulated strain. These data suggest the cumulative expression of TLR4, TLR2, and NOD1 recognition pathways and the requirement of neutrophils are essential for mucosal clearance of capsule-expressing, but not capsule-deficient, Hi.

**Discussion**

In this study, we investigated host immune components required for clearance of bacteria expressing capsular polysaccharide in vivo. We determined that the mucosal clearance of capsule-expressing Hib required the function of both TLR and NOD pathways, whereas individual deficiencies in each of these signaling cascades did not affect clearance of nonencapsulated Hi. Moreover, recruitment of neutrophils to the site of infection was essential for clearance of encapsulated Hib and ex vivo phagocytic killing of Hib required expression of the NOD1 signaling pathway. Cumulatively, these data suggest a model in which both encapsulated and unencapsulated strains likely elicit innate immune pathways by similar mechanisms; however, capsule-expression provides an advantage to this organism by altering the requirements for host signaling pathways that effectively limit prolonged colonization. It is unlikely that capsule polysaccharide directly affects recognition by PRMs, since capsule expression impacts the effectiveness of three distinct signaling pathways involving TLR2, TLR4, and NOD1. A further implication is that redundancies within these innate immune pathways are sufficient to promote effective mucosal clearance of bacteria that do not express capsular polysaccharide and do not require phagocytic killing.

Cross-talk and redundant responses among TLR2 and TLR4 recognition receptors has been previously described (32–34) and encapsulated Hib has been shown to mediate activation of both pathways in vitro (35). TLR4-mediated pulmonary clearance of Hib and neutrophil influx to the lung has also been described (36). We found that clearance of encapsulated Hib was dependent upon expression of both TLR2 and TLR4 signaling pathways, each with distinct contributions to innate immunity. The absence of TLR4 expression resulted in an attenuation of clearance that was amplified by the depletion of neutrophils. In a separate study, we show that TLR4 appears to be particularly important in early epithelial responses to colonizing Hi (C. Beisswenger, E. S. Lysenko, and J. N. Wesier, manuscript submitted). In contrast, for TLR2, data in this report indicate that a major contribution to clearance may be in the activity but not recruitment of luminal neutrophils. 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**TLR4, TLR2, and NOD1 are not individually required for clearance of nonencapsulated Hi**

The observation that individual expression of TLR2, TLR4, and the NOD1 signaling pathways was required for effective mucosal clearance of Hib was unexpected. To determine whether this phenomenon was specific to this encapsulated strain, the role of TLR2, TLR4, and NOD1 individually in the clearance of an isogenic unencapsulated mutant strain (b−) and an unrelated nonencapsulated strain (NTHi) was investigated (Fig. 4B). Like the Hib strain (b+), both the unencapsulated mutant strain and a nonencapsulated strain showed limited colonization of WT mice. Contrary to what was observed with the encapsulated Hib strain, individual deficiencies in these recognition proteins were not sufficient to attenuate clearance of nonencapsulated strain H631. Similarly, no increase in colonization of the unencapsulated mutant strain (b−) of H636 was detected for mice deficient in TLR2, TLR4, or NOD1. Moreover, unlike the Hib strain, neutrophil depletion did not result in an increase in the colonization of the unencapsulated mutant strain or
resulted in the selective ability of Hi-expressing capsular polysaccharide to thwart host defense mechanisms and promote stable colonization within the murine nasopharynx. Moreover, it is interesting that this typical extracellular pathogen (Hib) can evoke the intracellular NOD1 signaling pathway, resulting in mucosal clearance.

Although previous studies have identified a role for NOD1-mediated effects of bacteria and cell wall components in vitro (for review see Refs. 41, 42), our understanding of the contribution of this PRM with regard to innate immune responses to infection in vivo is still incomplete. In our system, NOD1 was not a requirement for recruitment of neutrophils to the site of infection. Instead, NOD1 signaling enhanced the phagocytic activity of cell populations similar to those found recruited to murine nasal spaces following Hib inoculation. These data are supported by a previous study demonstrating that the NOD1 signaling pathway can respond to synthetic meso-diaminopimelic acid containing compounds or peptidoglycan of Hi to enhance the opsonophagocytic killing of another encapsulated bacterial pathogen, S. pneumoniae (18), further suggesting a role for NOD1 signaling in clearance of encapsulated bacteria. Characterization of the NOD1-dependent neutrophil antimicrobial activity that impacts killing of encapsulated bacteria is the subject of on-going investigation.

The mechanism of delivery of the bacterial peptidoglycan components to the intracellular compartment where NOD1 is located in this system remains unknown, although access to these cytoplasmic pathways may not be limited for professional phagocytes. It has been shown that the type IV secretion apparatus expressed by the Helicobacter pylori cag pathogenicity island mediates delivery of peptidoglycan to the host cell cytoplasm (43). Since expression of a putative type IV secretion system (T4SS) within the genomic island ICEHin1056 has recently been described for Hi (44), we determined whether the Hb strain used in our experiments expressed this T4SS. However, PCR amplification of T4SS-specific and island-specific genes was not observed for H636, suggesting that T4SS-mediated delivery of peptidoglycan is unlikely to account for NOD1 activation in our experiments (data not shown). Alternatively, production of a bacterial pore-forming toxin has been shown to permit intracellular access of peptidoglycan fragments (45). The expression of such toxins has not been described for Hi; although the possibility that production of these proteins by other members of microbial flora permits ligand access to the host cell cytoplasm cannot be excluded. In this regard, one potential explanation for the increase in Hib colonization of TLR2, TLR4, and NOD1-deficient mice is that these immune-deficient strains carry an altered microbial flora that results in an environment more permissive for Hi colonization. However, the fact that an increase in colonization was not observed universally for all Hi strains and that mice lacking effective adaptive immune responses exhibited effective clearance of Hib, suggest that these results are specific to innate immune recognition of encapsulated bacteria.

This study also indicates a direct role for neutrophil recruitment and/or activity in preventing extended colonization of encapsulated Hib. In our system, we examined the role of neutrophils in bacterial clearance by pretreating mice with the mAb RB6-8S5 to deplete cells expressing Ly-6G. A potential limitation of this method is that this Ab also recognizes non-neutrophil subpopulations of monococytes, dendritic cells, and macrophages (46). Cyto-

References


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