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Lipooligosaccharides Containing Phosphorylcholine Delay Pulmonary Clearance of Nontypeable *Haemophilus influenzae*▽

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Nontypeable *Haemophilus influenzae* (NTHi) causes pulmonary infections in patients with chronic obstructive pulmonary disease and other mucociliary clearance defects. Like many bacteria inhabiting mucosal surfaces, NTHi produces lipooligosaccharide (LOS) endotoxins that lack the O side chain. Persistent NTHi populations express a discrete subset of LOS glycoforms, including those containing phosphorylcholine (PCho). In this study, we compared two NTHi strains with isogenic mutants lacking PCho for clearance from mice following pulmonary infection. Consistent with data from other model systems, populations of the strains NTHi 2019 and NTHi 86-028NP recovered from mouse lung contained an increased proportion of PCho$^+$ variants compared to that in the inocula. PCho$^-$ mutants were more rapidly cleared. Serial passage of NTHi increased both PCho content and bacterial resistance to clearance, and no such increases were observed for PCho$^-$ mutants. Increased PCho content was also observed in NTHi populations within non-endotoxin-responsive C3H/HeJ and Toll-like receptor 4 null (TLR4$^{-/-}$) mice, albeit at later times postinfection. Changes in bacterial subpopulations and clearance were unaffected in TLR2$^{-/-}$ mice compared to the subpopulations in and clearance from mice of the parental strain. The clearance of PCho$^-$ mutants occurred at earlier time points in both strain backgrounds and in all types of mice. Comparison of bacterial populations in lung tissue cryosections by immunofluorescent staining showed sparse bacteria within the air spaces of C57BL/6 mice and large bacterial aggregates within the lungs of MyD88$^{-/-}$ mice. These results indicate that PCho promotes bacterial resistance to pulmonary clearance early in infection in a manner that is at least partially independent of the TLR4 pathway.

Nontypeable *Haemophilus influenzae* (NTHi) is a human-specific commensal of the nasopharynx and upper airways. In contrast to encapsulated *H. influenzae* strains that cause invasive disease, NTHi strains are genetically diverse and aclonal (34). During normal carriage, NTHi causes no overt pathology and may in fact provide an immune stimulus that promotes the containment of other organisms (25). When mucociliary clearance is impaired, NTHi can cause opportunistic infections that include sinusitis, bronchitis, and otitis media (11). NTHi is also a major cause of infections associated with chronic obstructive pulmonary disease (COPD) (9, 14, 31), which is one of the most prevalent diseases affecting adults worldwide (28). Patients with COPD are colonized in their upper and lower airways with NTHi and other bacteria, which may persist for months or even years; changes in the subpopulations of bacteria within the COPD patient lung can be a determinant of the progression and severity of disease (41, 42, 44).

Clearance of NTHi bacteria from the lung is mediated by both innate and adaptive immune defenses. NTHi elicits a robust antibody response directed against a number of different surface moieties, and a substantial number of studies indicate that at least a subset of these antibodies may confer protection and/or bacterial clearance (1, 4, 8, 19, 21, 29, 33, 35, 36, 38, 66). Additional data indicate that cell-mediated immunity may also be important in the clearance or containment of NTHi infection in COPD patients (20).

As is true for many opportunistic organisms, the innate host response directed against *H. influenzae* bacteria and their components initiates bacterial clearance from the airway (58). Like most gram-negative bacteria, *H. influenzae* produces endotoxin that is predominantly hexa-acylated (23) and evokes host cell responses via Toll-like receptor 4 (TLR4) (22, 37). *H. influenzae* bacteria also produce proteins and lipoproteins that are recognized by TLR2 (6, 12, 45, 46, 57). Recent work indicates that intact *H. influenzae* bacteria also activate the TLR9 pathway, in addition to the TLR2 and TLR4 pathways (30). The central role of TLR4 activation in the pulmonary clearance of *H. influenzae* has been well established (58) and is dependent on the MyD88-dependent host cell signal pathway (65).

H. influenzae endotoxins are lipooligosaccharides (LOS) that lack the repeating O side chains typical of enteric bacteria (39). Instead, *H. influenzae* produces a highly diverse assortment of LOS glycoforms. The composition and structure of the *H. influenzae* LOS constantly shift due to temporal regulation and phase variation of genes involved in its assembly (40, 49, 59). LOS oligosaccharides contain epitopes that are also found on host cells, and thus NTHi is thought to persist via host mimicry that may blunt immune clearance (32). One of the host structures found in the *H. influenzae* LOS is phosphorylcholine (PCho), which is scavenged from host cells via the GlpQ phospholipase (10) and added to a discrete subset of LOS acceptors (26, 62). Prior work has shown that PCho confers a number of persistence-related phenotypes on NTHi bacteria, including host cell adherence and invasion (47, 50) and resistance to

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TABLE 1. Bacterial strains and phenotypes

Bacterial strain	Description	Reference(s)
NTHi 2019	Bronchial-patient isolate	7
NTHi 2019 *licD*	PCho$^-$ mutant	47
NTHi 86-028NP	Otitis media patient isolate	5
NTHi 86-028NP *licD*	PCho$^-$ mutant	15, 64

some host-derived antimicrobials (27). Recent work from our laboratory also shows that persistence in biofilm communities results in an increased PCho content of NTHi endotoxin and diminished host cell responses, presumably by affecting the TLR4 pathway (16, 64). In this study, we compare the levels of clearance of isogenic NTHi strains with and without PCho from the mouse lung following pulmonary infection. The results indicate that variants expressing PCho are better able to resist clearance from the mouse lung than mutants lacking PCho. Serial passage increased both PCho content and the length of bacterial persistence, and both phenotypes were lacking in PCho$^-$ mutants. Similar effects of PCho on persistence were observed in C3H/HeJ non-endotoxin-responsive mice. Thus, we conclude that PCho blunts the pulmonary clearance of NTHi by innate host defenses, albeit in a manner that may not be strictly TLR4 dependent.

MATERIALS AND METHODS

Bacteria. The NTHi strains used in this study are listed in Table 1. All NTHi strains were cultured at 37°C on brain heart infusion (BHI) agar (Difco) supplemented with 10 μg/ml hemin chloride (ICN) and 10 μg/ml β-nicotinamide adenine dinncleotide (Sigma). Hereinafter, this medium is referred to as supplemented BHI (sBHI) agar.

To prepare the inocula, bacteria were grown on sBHI agar plates overnight, suspended in sterile phosphate-buffered saline (PBS), and diluted to an optical density at 600 nm of 0.150 (1×10^8 CFU/ml). The inoculum concentration was confirmed by standard colony plate counting.

Mice. C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) or from the mouse repository at the National Cancer Institute (Bethesda, MD). C3H/HeN mice were purchased from the NCI. C3H/HeJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Breeding pairs of MyD88$^{-/-}$ (2), TLR2$^{-/-}$ (52), and TLR4$^{-/-}$ (17) mice were generously provided by Shizuo Akira via Elizabeth Hiltbold (Department of Microbiology and Immunology, WFUHS). All mice were 8 to 10 weeks of age and were housed under pathogen-free conditions.

Mouse lung infection. The mice were anesthetized by intraperitoneal injection with 2,2,2-tribromoethanol (Avertin) and infected intratracheally with $\sim 3 \times 10^6$ CFU of bacteria. At various times postinfection, the mice were anesthetized with 2,2,2-tribromoethanol and then euthanized by cervical dislocation. After the lungs were exposed, the pulmonary vascular system was flushed via the right ventricle with sterile PBS. The left lung of each mouse was homogenized, serially diluted, and spread onto sBHI agar plates, which were incubated overnight for plate counts. For each mouse, the right lung was infused with 4% paraformaldehyde for histopathologic analyses.

Paraffin sections and histopathology analysis. Fixed tissue specimens were dehydrated and embedded in paraffin. Sections (5 μm) were cut from paraffin-embedded blocks on a microtome and mounted from warm water (40°C) onto adhesive microscope slides. After serial deparaffinization and rehydration, tissue sections were stained with hematoxylin and eosin for histopathology assessment.

Cryosectioning and immunofluorescence staining. Fixed tissue samples were rinsed with 1× PBS at room temperature and placed into Cryomold (Sakura Finetek USA, Torrance, CA). Octyldecyl silane compound (Sakura Finetek USA, Torrance, CA) was added, and the blocks were frozen at −70°C for 1 h. Serial 5-μm sections were cut with an Accu-Edge low-profile blade (Feather Safety Razor, Japan) at −20°C and stored at −70°C. Immunofluorescence staining was performed using rabbit antisera recognizing NTHi and anti-PCho antibody, essentially as described previously (47).

LOS analysis. From lung homogenate bacterial isolates, LOS was isolated using a modified proteinase K procedure (3, 18). Briefly, bacteria were harvested from sBHI agar plates from the lung homogenates after 24 h of incubation, diluted to an optical density at 650 nm of 0.90 (1×10^9 CFU/ml) in sterile PBS, pelleted, and then lysed in 2.0% sodium dodecyl sulfate (SDS), 10 mM EDTA, 0.06 M Tris (pH 6.8). After overnight treatment with 2.5 μg/ml proteinase K (Sigma), the lysates were boiled for 5 min and digested overnight with 10 units staphylococcal nuclease (Sigma). LOS was precipitated with sodium acetate-ethanol, dialyzed overnight, and lyophilized. LOS was analyzed by Tricine-SDS-polyacrylamide gel electrophoresis (PAGE) (24) and visualized by ammonia silver staining (56). PCho was measured by immunoblotting with anti-PCho monoclonal antibody TEPC-15 (Sigma) or HAS (Statens Serum Institut).

Data analysis. Statistical analyses of the bacterial counts were performed using the nonparametric Mann-Whitney U test. As per standard practice, data sets for which P values of ≤ 0.05 were obtained were deemed significantly different.

RESULTS

NTHi mutants lacking PCho are readily cleared from mouse lungs. It has long been appreciated that *H. influenzae* populations in vivo are enriched for PCho$^+$ variants (16, 55, 60, 61). We used a mouse pulmonary-infection model to determine whether PCho affects the pulmonary persistence and/or the clearance of NTHi. The levels of clearance of two well-characterized NTHi strains (NTHi 2019 and NTHi 86-028NP) after intratracheal infection were compared with those of isogenic *licD* mutants lacking PCho (Fig. 1). The results show that significantly fewer *licD* bacteria than bacteria of the parental NTHi 2019 strain were recovered 48 h postinfection (Fig. 1A). Similarly, significantly fewer NTHi 86-028NP *licD* bacteria than bacteria of the parental strain were recovered from lung homogenates at 24 and 48 h postinfection. Notably, there were significant differences in the kinetics of clearance of the two strains following infection, which is similar to the well-documented differences in the levels of persistence/clearance of individual NTHi strains in patient carriage studies (41, 43) and in our recently published work with the chinchilla model of otitis media (15). The results also show a significant increase in the clearance of PCho$^-$ *licD* mutants of both strains. Thus, we conclude that PCho delays the clearance of NTHi in pulmonary infections. As NTHi populations contain various percentages of PCho$^+$ and PCho$^-$ variants, we next asked whether lung carriage resulted in an increased number of PCho$^+$ variants compared to the number in the inocula. LOS was purified from inocula and mouse-passaged bacteria and analyzed for PCho content by Tricine-SDS-PAGE and immunoblotting. The data show that the amount of LOS PCho increases in recovered NTHi 2019 and NTHi 86-028NP bacteria at 24 and 48 h postinfection (Fig. 1, insets). These results are consistent with the hypothesis advanced by several groups that selective pressure within the lung may favor NTHi variants with PCho$^+$ LOS forms (15, 55, 60, 61).

Histopathologic analysis of lung tissue from infected mice. Lungs from infected mice were sectioned and stained for histopathology assessment (Fig. 2). The sections were examined blind as sets by a trained veterinary pathologist (N. Kock). Airway epithelia were examined for signs of membrane damage, apoptotic cells, and vesicle formation. Edema was scored based on the size of the affected area, and cellular infiltration was assessed. For each lung, an overall semiquantitative inflammatory score was assessed by compiling all of the criteria. The results showed a greater overall inflammation score for

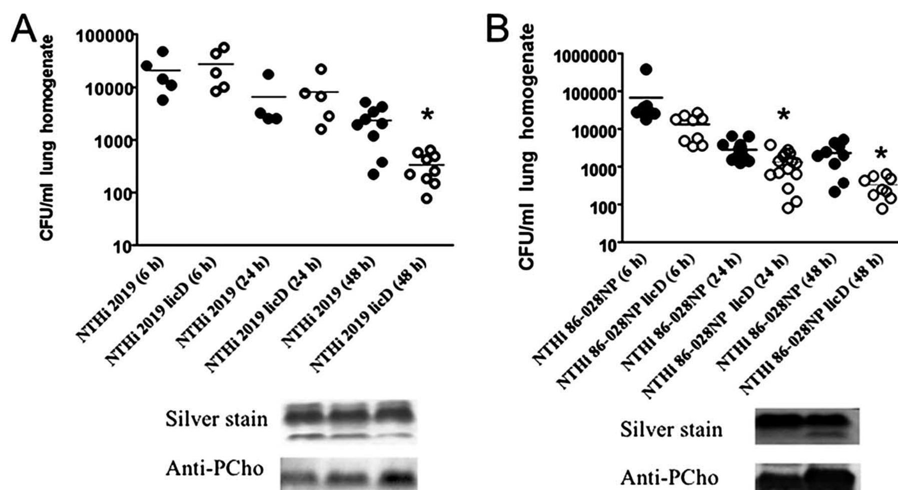

FIG. 1. PCho^{-} mutants of NTHi are readily cleared from mouse lungs. Symbols represent numbers of CFU recovered from individual mouse lungs after infection with NTHi 2019 or NTHi 2019 *licD* (A) and NTHi 86-028NP or NTHi 86-028NP *licD* (B). Statistical means are shown as horizontal bars. An asterisk indicates a significant difference from the value for the wild-type strain. LOS was purified from NTHi 2019 or NTHi 86-028NP bacteria in the inoculum; bacteria were recovered from C57BL/6 mouse lungs at 24 h and 48 h postinfection and analyzed by Tricine-SDS-PAGE, followed by silver staining and immunoblotting as described in Materials and Methods.

animals infected with the *licD* mutant strains at the earliest time point (6 h) postinfection than those for animals infected with parental strains (Fig. 2). No other significant differences were noted. Immunofluorescent staining of lung cryosections showed diffuse distribution of individual bacteria within the lung at the earliest time points, with little staining observed thereafter (data not shown).

Repeated passage increases the resistance of NTHi 86-028NP to clearance from mouse lungs. Because the passage of NTHi in mouse lungs enriches for PCho^{+} variants, we reasoned that if PCho promotes resistance to clearance, then the length of NTHi persistence in the mouse lung would increase with serial passage in accordance with PCho content. NTHi 86-028NP bacteria (mouse passaged) recovered from the first round of mouse lung infection as well as NTHi 86-028NP bacteria (original stock) that had not been passaged through a mouse lung were used as inocula for another round of mouse lung infection. The mice infected with similarly passaged NTHi 86-028NP *licD* served as controls. The data show that there were significantly more NTHi 86-028NP bacteria recovered from the repeated passage than from the original stock. However, no such increase in bacterial numbers was observed with the mouse-passaged NTHi 86-028NP *licD* bacteria (Fig. 3). Analysis of LOS purified from these bacteria showed an increase in PCho content coinciding with increased passage (Fig. 3B). We thus conclude that carriage results in NTHi populations that are more resistant to host clearance, in accordance with increased PCho content.

PCho increases the resistance of NTHi to clearance from mouse lung mediated by TLR4 in vivo. The host response to *Haemophilus influenzae* in the lung is largely mediated by TLR4 (58, 65), and our recent work shows that the growth of NTHi in biofilms results in diminished LOS bioactivity in conjunction with increased PCho content (64). Thus, we asked whether the increased resistance to clearance associated with PCho is directly related to TLR4. NTHi clearance was compared in endotoxin-responsive (C3H/HeN) and endotoxin-nonresponsive (C3H/HeJ) mice. In accordance with prior

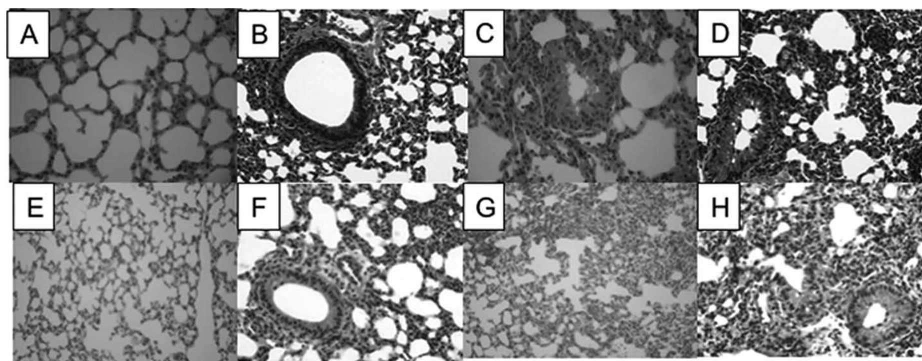

FIG. 2. Histopathologic analysis of infected mouse lung tissue. Sections were stained and scored in a blind fashion for inflammatory markers as described in Materials and Methods. (A to D) Light micrographs of hematoxylin- and eosin-stained paraffin sections from mice infected with NTHi 2019 (A and B), NTHi 2019 *licD* (C and D), NTHi 86-028NP (E and F), or NTHi 86-028NP *licD* (G and H).

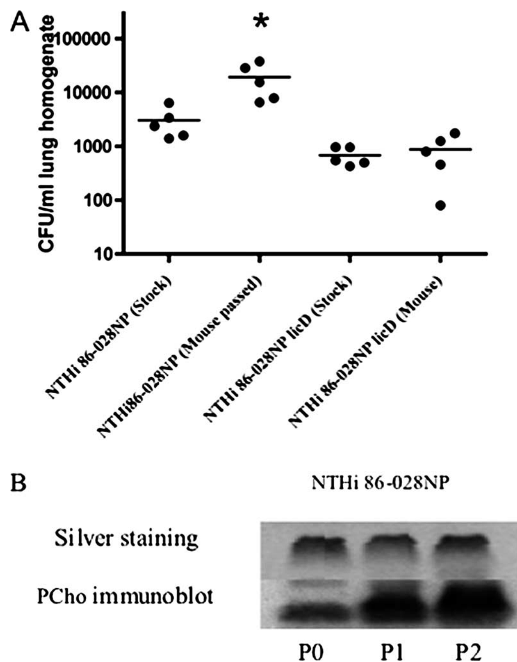

FIG. 3. Repeated passage increases the resistance of NTHi to clearance from mouse lung. (A) Comparison of CFU counts from mice infected with NTHi 86-028NP before and after mouse passage. Horizontal bars represent the statistical means. The asterisk denotes statistically significant differences. (B) Comparison of PCho contents in LOS from mouse-passaged strains and stock strains. LOS was purified and analyzed as described for the preceding figures.

work, higher counts of NTHi bacteria were observed in the lungs of C3H/HeJ mice (Fig. 4A) than in the lungs of C3H/HeN mice (Fig. 4B). In the C3H/HeN mice, we observed significant differences in the levels of clearance of the parental NTHi strain and the *licD* mutant (Fig. 4B), which were consistent with the infection studies performed using C57BL/6 mice (Fig. 1). However, the only significant differences in CFU counts from C3H/HeJ mice were observed at later time points (Fig. 4A). Analysis of LOS purified from the NTHi bacteria revealed that the proportion of $PCho^+$ bacteria increased even in the absence of the TLR4 response, albeit at later time points (Fig. 4C and D). Comparable results were obtained using $TLR4^{-/-}$ mice (data not shown). In parallel experiments, we saw no difference in either CFU counts or the magnitude or timing of the shift in $PCho^+$ subpopulations between $TLR2^{-/-}$ mice and mice of the parental strain (data not shown).

NTHi persistence in $MyD88^{-/-}$ mouse lungs. The data indicated that the increase in $PCho^+$ variants was temporally related to the inflammatory response. Prior studies showed that *H. influenzae* clearance was severely impaired in $MyD88^{-/-}$ mice (65). Therefore, if our hypothesis that the inflammatory response promotes the observed increase in $PCho^+$ subpopulations, then a $PCho^-$ mutant should have no defect in this mouse background. Comparison of levels of clearance of NTHi 2019 and NTHi 2019 *licD* from C57BL/6 mice and isogenic $MyD88^{-/-}$ mice revealed that the difference in clearance associated with the loss of PCho was absent in the absence of MyD88 (Fig. 5A). Likewise, there was no difference in the markers of inflammation in sections of lung tissue from $MyD88^{-/-}$ mice infected with NTHi 2019 and NTHi 2019 *licD* (data not shown).

We next analyzed cryosections of infected lung tissue from

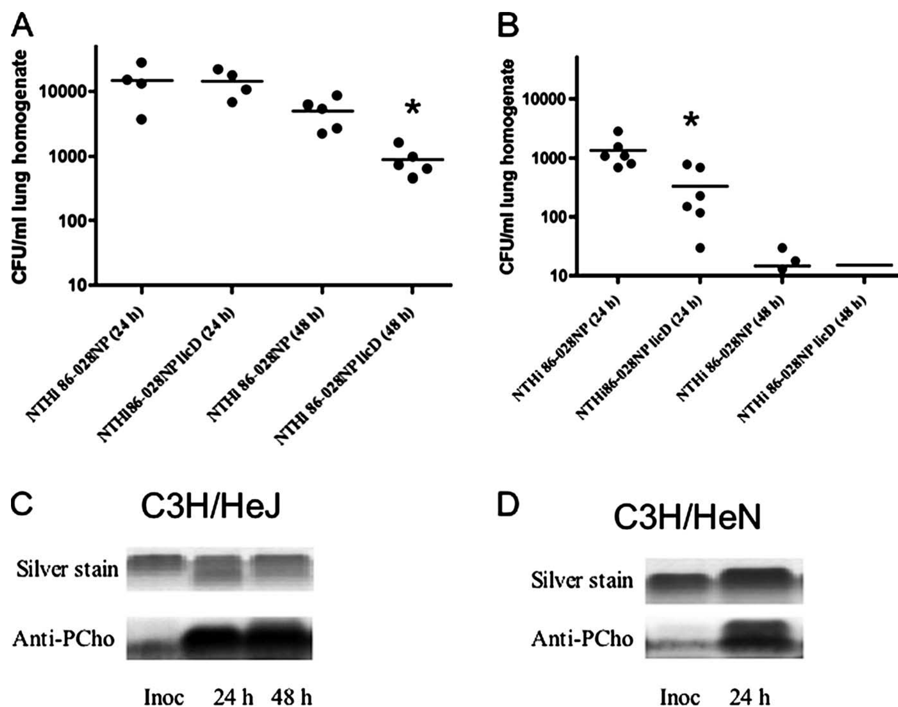

FIG. 4. Role of TLR4 in PCho-related NTHi clearance resistance. Graphs depict numbers of bacterial CFU recovered from the infected lungs of C3H/HeJ (A) or C3H/HeN (B) mice. Asterisks denote statistically significant differences. The PCho contents of purified LOS are shown for bacteria recovered from C3H/HeN mice (C) or C3H/HeJ mice (D) at 24 and 48 h postinfection. Inoc, inoculum.

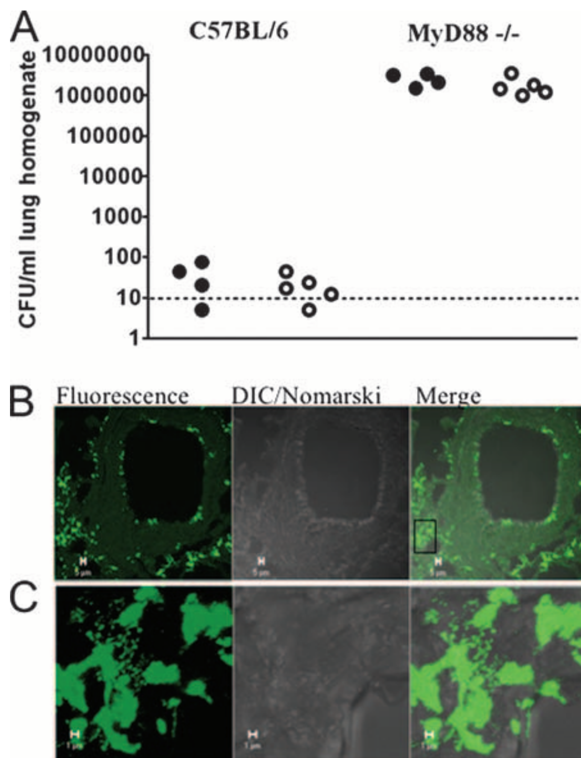

FIG. 5. NTHi persistence in MyD88$^{-/-}$ mice. C57BL/6 mice and isogenic MyD88$^{-/-}$ mice were infected intratracheally with NTHi 2019 (filled circles) or NTHi 2019 *licD* (open circles). At 72 h postinfection, mice were euthanized and their lungs were excised. (A) CFU counts from lung homogenates from infected mice. (B) Representative images obtained following immunofluorescent staining of NTHi bacteria (green) within cryosections of lung tissue of MyD88$^{-/-}$ mice. Multicellular communities were readily visible throughout the lung. DIC, differential inference contrast optics. (C) Higher-magnification image of the area in the boxed region in panel B. Bars in panels B and C indicate 5 and 1 μm, respectively.

MyD88$^{-/-}$ mice by immunofluorescent microscopy (Fig. 5B and C). At 72 h postinfection, we observed multicellular bacterial communities within the lung tissue. No such communities were observed in infected lungs from C57BL/6 mice.

DISCUSSION

As a commensal, NTHi is highly adapted to resist host clearance and persist in the airways. Our prior work showed that LOS modifications occurring in vivo, such as sialylation and the addition of PCho, impact a variety of persistence-associated bacterial phenotypes that include adherence to and invasion of airway cells and dampening of the inflammatory response (47, 48, 50, 51, 64). In this study, we sought to address how PCho content affects bacterial persistence in the lung. Mouse pulmonary infections are a well-established model system for airway persistence/clearance for many organisms, including NTHi (53, 54). Thus, we compared the levels of clearance of two different NTHi strains and their isogenic *licD* mutants from the mouse lung. The data show that carriage in vivo enriches for PCho$^+$ variants (Fig. 1), as has been well established in patient studies and with animal models (16, 55, 60, 61). Moreover, our data show that serial passage confers an enhanced persistence phenotype on NTHi and that this is lacking in PCho$^-$ mutants (Fig. 3). These results are consistent with our recently published work showing that PCho$^-$ mutants of NTHi have a defect in biofilm formation within the chinchilla middle ear (15, 16).

Additional information gleaned from the infection studies using mutant mice includes the finding that the shift to PCho$^+$ variants and the enhanced persistence of PCho$^+$ variants compared to PCho$^-$ mutants were delayed in non-endotoxin-responsive mice (Fig. 4). These findings link the fitness advantage of PCho$^+$ variants to host responses to LOS early in infection, which is consistent with our recent observation that PCho blunts host inflammatory responses to NTHi LOS and bacteria (15, 64). The contribution of PCho to the colonization and persistence of *H. influenzae* is multifactorial. In addition to having anti-inflammatory effects, PCho adheres to host cells, which is mediated by its binding to the platelet-activating factor receptor (13, 47, 50), it enhances resistance to some host antimicrobials (27), and it promotes biofilm formation both in vitro and in vivo (16).

Our infection studies using TLR2$^{-/-}$ mice do not support a significant role for this pathway in NTHi persistence or in the shift in variants expressing PCho. These data are consistent with findings from other groups (65) and support the conclusion that the TLR4 response is important in the early innate response to NTHi infection. These data are also consistent with prior work showing that TLR4 and TLR2 responses had different temporal roles in innate defenses against *Salmonella enterica* serovar Typhimurium infection in mice (63).

In summary, this study clarifies the role of a surface modification common to many mucosa-adapted pathogens in bacterial persistence in vivo. Ongoing work in our laboratory is devoted to more fully defining the adaptive strategies used by NTHi to persist in the airways. As NTHi causes opportunistic infections that are a major public health problem, defining how the organism persists in vivo is an essential step in learning to better prevent and/or manage these infections.

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