Immunity to the Conserved Influenza Nucleoprotein Reduces Susceptibility to Secondary Bacterial Infections


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Influenza causes >250,000 deaths annually in the industrialized world, and bacterial infections frequently cause secondary illnesses during influenza outbreaks, including pneumonia, bronchitis, sinusitis, and otitis media. In this study, we demonstrate that cross-reactive immunity to mismatched influenza strains can reduce susceptibility to secondary bacterial infections, even though this fails to prevent influenza infection. Specifically, infecting mice with H3N2 influenza before challenging with mismatched H1N1 influenza reduces susceptibility to either Gram-positive Streptococcus pneumoniae or Gram-negative Klebsiella pneumoniae. Vaccinating mice with the highly conserved nucleoprotein of influenza also reduces H1N1-induced susceptibility to lethal bacterial infections. Both T cells and Abs contribute to defense against influenza-induced bacterial diseases; influenza cross-reactive T cells reduce viral titers, whereas Abs to nucleoprotein suppress induction of inflammation in the lung. These findings suggest that nonneutralizing influenza vaccines that fail to prevent influenza infection may nevertheless protect the public from secondary bacterial diseases when neutralizing vaccines are not available.

Secondary bacterial infections often follow influenza infection and can lead to a variety of illnesses including pneumonia, bronchitis, sinusitis, and otitis media (1, 2). Secondary bacterial pneumonia is a particularly serious consequence of influenza infection. It was the primary cause of death during the 1918 influenza pandemic (3) and was associated with significantly higher morbidity and mortality during the 2009 pandemic (4).

Vaccines are the mainstay of public-health efforts to prevent influenza epidemics. These vaccines aim to prevent infection by eliciting neutralizing Abs that bind the hemagglutinin and neuraminidase proteins on the surface of influenza virions. Unfortunately, mutations and reassortments in the surface proteins of influenza viruses allow new strains to emerge and evade neutralizing Abs (5, 6). Consequently, each year, new vaccines are produced to “match” the most dangerous contemporary strains.

In animal models, mismatched influenza vaccines can prime nonneutralizing immunity that speeds viral clearance and reduces mortality, despite failing to prevent infection (7, 8). Mismatched vaccines may not prevent human pandemics, but they might lessen their severity when matched vaccines are not available. Indeed, several studies suggest humans may benefit from nonneutralizing immunity to influenza (9–12). A recent study demonstrated that the presence of influenza-specific memory in humans correlates with nonneutralizing immunity that significantly reduces the severity of illness (13). These researchers postulated that CD4 T cells confer protection by improving Ab responses to conserved internal viral proteins (13). Unfortunately, many factors confound the interpretation of human studies of influenza, and public-health campaigns to date have largely neglected the potential for nonneutralizing immunity to combat influenza outbreaks or the associated increase in secondary bacterial infections.

Data from mouse models suggest influenza infection increases susceptibility to secondary bacterial infections by suppressing neutrophil function, decreasing mucociliary flow, desensitizing innate immunity, and creating favorable environments for bacterial adherence and colonization (1). Cytokines, including ILs and IFNs, also affect susceptibility (14–16), suggesting that ongoing immune responses to influenza may facilitate bacterial colonization of the lung. In this study, we investigate whether nonneutralizing, mismatched immunity to influenza impacts susceptibility to secondary bacterial infections.

Materials and Methods

Mice

Wild-type and B cell-deficient μMT C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Ab-deficient AID-μS C57BL/6 mice were described previously (17, 18). All mice were bred in the specific pathogen-free Trudeau Institute Animal Breeding Facility after embryo rederivation. Experimental mice were matched for age and sex and cared for according to Trudeau Institute guidelines. Recumbent mice, and mice that lost more than 30% weight, were considered moribund and euthanized.

Viruses

Influenza virus A/HKx31 (H3N2), influenza virus A/PR/8/34 (H1N1), cold-adapted (ca) influenza virus c.a.A/Alaska/72/CR9 (caH3N2), and the...
The intranasal median lethal dose of strain 6304 was confirmed by plating. The intranasal median lethal dose of strain 6304 was determined by real-time PCR measuring acid polymerase copy number.

**Bacteria**

Serotype 4 *Streptococcus pneumoniae* (ATCC strain 6304) grown on blood agar plates was used to inoculate tryptic soy broth cultures, which were grown at 37°C without shaking in sealed tubes. After dilution to an OD<sub>600nm</sub> of 0.15, they were regrown to an OD<sub>600nm</sub> of 0.45 and washed with saline, and ~250 CFU were applied in a volume of 50 μl saline to the nares of lightly anesthetized mice. The number of bacteria in the inoculating dose was confirmed by plating. The intranasal median lethal dose of strain 6304 is ~1.5 x 10^8 CFU when grown as described above and administered to naive mice. Additional studies also used serotype 3 *S. pneumoniae* strain URF918 (23) and *Klebsiella pneumoniae* strain IA565 (24). Innocula of strain URF918 were prepared as described for ATCC strain 6304. Working stocks of strain IA565 were prepared by growing in tryptic/soy broth to log phase (OD<sub>600</sub> = 0.900), adding 20% glycerol and freezing aliquots at −70°C. To prepare inocula, 100 μl working stock was streaked on tryptic/soy agar plates, grown overnight at 37°C, and scraped to seed tryptic/soy broth cultures at OD<sub>600</sub> = 0.050. Cultures were grown in 37°C incubator/shaker at 180 rpm for ~2 h to log phase (OD<sub>600</sub> = 0.900), spun down, and resuspended in PBS to a concentration of 10^8 CFU per 50-μl infection dose.

**Treatments**

T cell depletions were performed as described previously (19). The depletion protocols removed >90% of the targeted cells from the spleen and bronchoalveolar lavage fluid, as determined by flow cytometric analyses of Ab-treated animals that were euthanized at day 5 after H1N1 infection (data not shown). Recombinant A/PR/8/34 influenza nucleoprotein (NP) was generated as a C terminal histidine-tagged protein in *Escherichia coli* and isolated using the ProBond system (Invitrogen), as described previously (17). Immunizations contained 30 μg NP and used 20 μg *E. coli* serotype 0111: B4 LPS (Enzo Life Sciences) plus alum as adjuvant (17). H3N2 immune serum was collected 21 d postinfection with H3N2, and 350 μl was transferred to naive mice by i.p. injection on the day prior to H1N1 challenge. Passive immunization with mouse IgG2a NP-specific mAb H16-L10-4R5/HB-65 (25) was achieved by administering 350 μg i.p. injections on the day of and the day prior to H1N1 challenge. Control mice received serum from naive mice or isotype-matched mAb C1.18.4. All mAbs were protein G purified and supplied by BioXCell, who reported ~2 endotoxin units/mg.

**Statistics**

Survival curves were analyzed by log-rank tests. CFU and viral titer data that fell below the limit of detection were assigned a value below that limit and, thus, were analyzed by nonparametric Mann–Whitney or Kruskal–Wallis tests. Bacteremia was scored positive or negative and analyzed by χ² tests.

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**FIGURE 1.** Long-term cross-reactive immunity to influenza protects from secondary bacterial infection. (A) General experimental approach followed for all studies; see each figure legend for specifics of treatments and timing. For (B)–(E), C57BL/6 mice were infected with H3N2 influenza or left uninfected. After 5–6 mo, the mice were challenged with H1N1 influenza, followed 5, 7, or 14 d later by infection with *S. pneumoniae* (Spn). (B) Survival of mice challenged with Spn on day 5 after H1N1 infection (n = 10 mice/group). Mice infected previously with H3N2 showed significantly greater survival than control mice (p = 0.006 by log-rank test). (C) Bacterial burden in the lung 24 h after Spn infection. Mice exposed previously to H3N2 harbored significantly fewer bacteria than control mice when both groups were infected with Spn at days 5, 7, and 14 after H1N1 infection (all p < 0.04 by Mann–Whitney U test). Although susceptibility peaked at day 7, we focused survival studies on day 5 because H1N1-infected naive mice showed significantly greater weight loss than H3N2 immune mice on days 7 and 14 but not on day 5 (data not shown). (D) Bacterial burden in the blood 24 h after Spn infection. (E) Influenza burden at the time of Spn infection. Mice exposed previously to H3N2 harbored significantly less virus than control mice at days 5 and 7 after H1N1 infection (both p < 0.02 by Mann–Whitney U test). The day 5 data and day 7/14 data were collected in two separate H1N1/Spn challenge studies using a single cohort of H3N2-exposed animals; dotted lines depict limit of detection.
Results
Nonneutralizing immunity to influenza protects from secondary bacterial infection

Fig. 1A depicts our general experimental approach to assessing the impact of prior immunity to influenza on susceptibility to secondary bacterial infection. Naive mice readily survived low-dose intranasal challenge with 250 CFU S. pneumoniae (Fig. 1B). Naive mice also survived low-dose intranasal challenge with H1N1 influenza (Fig. 1B). However, consistent with prior reports (26), we observed that mice succumbed to bacterial infection when challenged with low-dose S. pneumoniae following a sublethal influenza challenge (Fig. 1B). To investigate the impact of nonneutralizing, mismatched immunity to influenza, we infected mice with low-dose H3N2 influenza, challenged 5–6 mo later with low-dose H1N1 influenza, and then measured susceptibility to S. pneumoniae. We observed that prior exposure to H3N2 influenza improved survival (Fig. 1B), reduced pneumococcal colonization of lung tissue (Fig. 1C), and largely prevented bacteremia (Fig. 1D). Notably, susceptibility to bacterial infection did not simply correlate with viral titers at the time of challenge. For example, mice challenged with S. pneumoniae on days 5 and 14 after H1N1 infection exhibited similar bacterial burden (Fig. 1C), despite more than a 10,000-fold difference in viral titers at those time points (Fig. 1E).

To investigate the specificity of the H3N2-induced protection from secondary bacterial infection, we evaluated protection conferred by Sendai virus, a parainfluenza virus that causes an acute pulmonary infection similar to influenza but does not prime cross-reactive immunity to influenza (20). In parallel, we examined protection conferred by a caH3N2 vaccine strain (19, 21, 27, 28). We found that exposure to either the H3N2 influenza virus or the live attenuated caH3N2 vaccine protected against H1N1-induced susceptibility to pneumococcal infection as early as 3 wk after exposure (Fig. 2A). The protection was associated with reduced bacterial burden in the lungs (Fig. 2B) and reduced H1N1 titers (Fig. 2C). In contrast, prior exposure to Sendai virus had no significant impact on H1N1-induced susceptibility to pneumococcal infection (Fig. 2A). Infection with Sendai virus, like H1N1 infection, induced susceptibility to S. pneumoniae when challenged on day 7 (29), but this susceptibility waned by 26 d postinfection (Fig. 3), indicating that residual impacts of primary Sendai infection did not account for the pneumococcal susceptibility observed when Sendai-exposed mice were infected with H1N1 influenza. Thus, specific cross-reactive immunity to influenza, not just conditioning of the lung by any viral infection, reduces susceptibility to secondary bacterial infection. Importantly, the cross-reactive immunity to influenza reduced susceptibility to diverse types of bacterial infections: the bacterial challenge studies described above used a serotype 4 strain of Gram-positive S. pneumoniae, but similar results were observed when mice were challenged with serotype 3 S. pneumoniae (Fig. 4A–C) or K. pneumoniae (Fig. 4D–F), a Gram-negative bacterium.

It has been shown that nonneutralizing immunity to influenza can accelerate viral clearance (7, 8, 30). Thus, preexisting immunity to influenza may have shifted the period of H1N1-induced susceptibility to pneumococcal infection, such that mice became suscep-
tible prior to day 5 after H1N1 challenge. To investigate this possibility, we examined the kinetics of susceptibility in greater detail. We found that mice were susceptible to H1N1-induced pneumococcal infection when bacteria were administered on days 3, 5, and 7, but not day 1, after H1N1 infection, and that prior infection or vaccination with H3N2 suppressed pneumococcal susceptibility at these same times (Fig. 2D). Again, susceptibility correlated with increased bacterial burden (Fig. 2E) and higher viral titers (Fig. 2F). Thus, prior exposure to H3N2 influenza did not accelerate the time of susceptibility. Rather, preexisting mismatched immunity to influenza reduced overall susceptibility to pneumococcal infection.

Cross-reactive T cells and Ab contribute to protection

Cross-reactive CD8 T cells can facilitate nonneutralizing protection against mismatched influenza strains (7, 8), and influenza cross-reactive memory T cells produce IFN-γ, one of the cytokines that contributes to H1N1-induced susceptibility to pneumococcal infection (15). Thus, cellular immunity to influenza might be predicted to exacerbate susceptibility to pneumococcal disease. However, depletion of all T cells (anti-Thy1 treatment) or depletion of only CD8 T cells from H3N2 immune mice immediately prior to H1N1 challenge modestly diminished the protection conferred by prior exposure to H3N2 (Fig. 5A) and slightly elevated both the bacterial burden (Fig. 5B) and viral titer (Fig. 5C). Thus, the presence of cross-reactive memory T cells did not exacerbate pneumococcal infection and, rather, contributed to cross-reactive defense against bacterial disease, at least in part, by reducing H1N1 titers.

Abs to conserved viral proteins also contribute to cross-reactive immunity to influenza (7, 17, 18, 31, 32). To test the role of Ab in defense against H1N1-induced pneumococcal susceptibility, we administered H3N2 immune serum or control serum to naive mice prior to infection with H1N1 influenza. The mismatched H3N2 immune serum significantly decreased susceptibility to secondary pneumococcal infection (Fig. 5D). Despite conferring significant protection from lethality, passive immunization with mismatched serum did not significantly reduce bacterial burden (Fig. 5E) or...
viral titers (Fig. 5F) in the lung. However, H3N2 immune serum did significantly reduce levels of bacteremia and the number of mice with detectable bacteria in blood cultures (Fig. 5E).

Influenza cross-reactive nonneutralizing immunity typically recognizes conserved, internal proteins of influenza, and immunity to NP, a highly conserved internal protein, confers significant protection from lethal influenza challenge (7, 8, 17, 18, 32–38). We observed that vaccinating mice with purified recombinant NP conferred robust protection from H1N1-induced susceptibility to secondary pneumococcal infection (Fig. 5G). NP vaccination markedly reduced pneumococcal burden (both $p < 0.003$ by log-rank test) and decreased the pulmonary bacterial burden ($p = 0.004$ by Mann–Whitney; $n = 15$ mice/group) but did not significantly impact viral burden. *Statistically significant difference, $p < 0.05$.

In comparison with H3N2-infected mice treated with control mAb, mice treated with anti-CD8 exhibited significantly decreased survival ($p = 0.04$ by log-rank test), and mice treated with anti-Thy1 showed significantly increased bacterial and viral burden (both $p < 0.05$ by Kruskal–Wallis test comparing all H3N2-infected mice). (D–F) C57BL/6 mice received passive immunizations with H3N2 immune serum or control serum. The next day, they were challenged intranasally with H1N1 influenza. After 5 d, all mice were challenged with Spn. (D) Survival ($n = 30$ mice/group; data pooled from three independent studies). Bacterial burden in the lung and blood 24 h after Spn infection (E) and influenza burden at the time of Spn infection (F). Passive immunization with H3N2 immune serum significantly increased survival ($p = 0.005$ by log-rank test) and decreased the incidence of bacteremia ($p = 0.003$ by $\chi^2$ test; $n = 10$ mice/group) but did not significantly impact pulmonary bacterial or viral burden. (G–I) C57BL/6 mice were infected with H3N2 influenza or immunized i.p. with recombinant NP (rNP) using LPS/alum adjuvant; controls were mock immunized with adjuvant alone. After 21 d, mice were challenged intranasally with H1N1 influenza, followed 5 d later with Spn. (G) Survival ($n = 20$ mice/group; data pooled from two independent studies). (H) Bacterial burden in the lung 24 h after Spn infection. Infection with H3N2 or immunization with rNP significantly increased survival (both $p < 0.0001$ by log-rank tests), decreased bacterial burden (both $p < 0.001$ by Kruskal–Wallis test), and decreased viral burden ($p < 0.0001$ and $p < 0.05$, respectively, by Kruskal–Wallis test). (J–L) C57BL/6 mice were passively immunized with NP-specific mAb or isotype-matched control mAb (Mouse IgG2a) and then challenged intranasally with H1N1 influenza. After 5 d, all mice were challenged with Spn. (J) Survival ($n = 40$ mice/group; data pooled from four independent studies). (K) Bacterial burden in the lung and blood 24 h after Spn infection. Infection with NP mAb significantly increased survival ($p = 0.0003$ by log-rank test) and decreased the pulmonary bacterial burden ($p = 0.004$ by Mann–Whitney; $n = 15$ mice/group) and incidence of bacteremia ($p = 0.003$ by $\chi^2$ test; $n = 15$ mice/group) but did not significantly impact viral burden. *Statistically significant difference, $p < 0.05$. 

Depletion of CD8 T cells alone, or both CD4 and CD8 T cells, at the time of H1N1 challenge did not significantly impair the protection conferred by NP vaccination (Fig. 6A), suggesting the involvement of alternative effector mechanisms. Other studies have
demonstrated that Abs to NP can confer significant protection from lethal influenza challenge (17, 18, 32). Consistent with a critical role for Abs, NP vaccination poorly protected B cell-deficient μMT mice and Ab-deficient AID,μS mice from death following influenza and S. pneumoniae infections (Fig. 6B and 6C). To definitively assess the protective capacity of NP-specific Abs, we passively transferred NP-specific mAb into naive mice and then challenged with influenza followed by S. pneumoniae. Remarkably, administration of NP-specific mAb conferred robust protection from H1N1-induced secondary pneumococcal disease (Fig. 5J). As with passive immunization with H3N2 immune serum, NP-specific mAb did not significantly reduce viral titers in the lung (Fig. 5L) but did reduce the number of bacteremic mice and levels of bacteremia (Fig. 5K). Notably, passive immunization with NP-specific mAb did not not not not significantly reduce bacterial infection directly because it did not affect the median lethal dose of S. pneumoniae in naive mice (data not shown) and only reduced pneumococcal lethality in mice infected previously with influenza.

Immunity to NP reduces lung inflammation

NP-specific Abs can help to reduce H1N1 titers in lethal influenza challenge models (17, 18, 31, 32) but did not appear to reduce H1N1 titers in our model of sublethal influenza challenge. Abs are known to play diverse roles during host defense, including the suppression of inflammation (39). Indeed, in our studies, passive immunization with NP-specific mAb markedly suppressed levels of inflammatory cytokines and chemokines in lung tissue of H1N1-infected mice, including IL-6, CCL2/MCP-1, and CXCL1/KC (Fig. 7B–D). Notably, levels of IFN-γ were not affected (Fig. 7A). Treatment with H3N2 serum likewise reduced levels of pulmonary inflammation, as did active vaccination with H3N2 virus or NP (Fig. 7B–D). In addition, the nonneutralizing immunity induced by infection, immunization, or Ab transfer also significantly reduced the expression of the platelet-activating factor receptor (Fig. 7E). Elevated expression of the platelet-activating factor receptor has been associated with enhanced inflammation and lung pathology during influenza infection (40, 41). These findings suggest non-neutralizing immunity, and cross-reactive NP-specific Abs, in particular, may reduce susceptibility to bacterial infection by reducing inflammation and pathological damage to lung epithelium, events that facilitate colonization of the lung by bacteria (1).

Discussion

Secondary bacterial infections are a common complication of influenza infection and cause significant morbidity and mortality (1, 2). Because of this important clinical problem, we have used a murine model to examine the influence of influenza infection on susceptibility to secondary bacterial infections and how this can be prevented. Using this model, we have shown that the susceptibility to secondary infection applies to several strains of bacteria and results in significant lung colonization, bacterial dissemination, and death. Consistent with prior studies (1, 14–16), we also found that the window of susceptibility to secondary infection begins at day 3 of influenza infection and extends until at least day 14, when the vast majority of virus has been cleared. These results suggest that susceptibility to secondary infection is not just due to influenza infection but may also be associated with the immune response to the virus, including inflammation in the lungs. In fact, it has been postulated that the extensive mortality observed in the 1918 influenza pandemic was the result of extensive immunopathology that increased susceptibility to secondary bacterial pneumonia (42).

This model has also allowed us to explore how this susceptibility to secondary infections can be overcome. The most common way to manipulate immunity to influenza is via vaccination. Neutralizing immunity to influenza, which is the goal of the yearly influenza vaccine, can prevent infection and illness. Although this is the most desirable goal of vaccination, it is not always possible because of rapid antigenic changes in the virus or the appearance of new viral strains. Prior studies have demonstrated that nonneutralizing immunity can reduce influenza illness and mortality in mice. Our observations demonstrate that nonneutralizing immunity to influenza also confers remarkable protection from secondary bacterial infections. Nonneutralizing immunity can be conferred by mis-

FIGURE 6. The immunity to influenza NP that protects from secondary bacterial infection is compromised in mice lacking B cells or circulating Ab, but not T cells. (A) Wild-type mice were immunized i.p. with recombinant NP (rNP) using LPS/alum adjuvant; controls were mock immunized with adjuvant alone. After 21 d, mice were challenged intranasally with H1N1 influenza, followed 5 d later with S. pneumoniae (Spn). On days 20 and 22, mice were treated with CD8 mAb or a combination of CD4 and CD8 mAb; controls received a rat IgG2b control mAb. Survival was not compromised significantly in mice treated with CD8 or CD4 and CD8 mAb (n = 8–10 mice/group). (B and C) Wild-type (WT) and B cell-deficient μMT mice (B) or circulating Ab-deficient AID,μS mice (C) were immunized i.p. with rNP using LPS/alum adjuvant; controls were mock immunized with adjuvant alone. After 21 d, mice were challenged intranasally with H1N1 influenza, followed 5 d later with Spn. Among mice immunized with rNP, WT mice showed significantly increased survival when compared with either μMT or AID,μS mice (both p < 0.0001 by log-rank tests; n = 10 mice/group).
FIGURE 7. Immunity to NP suppresses induction of inflammatory cytokines and chemokines. Levels of mRNA encoding IFN-γ (A), IL-6 (B), CCL2 (C), CXCL1 (D), and platelet-activating factor receptor (PAF-R) (E) in lung tissue from C57BL/6 mice infected with H1N1 influenza for 5 d. Graphs show the fold change (FC) from uninfected controls. As indicated, mice were previously exposed to H3N2 influenza, vaccinated with recombinant NP (rNP), passively immunized with H3N2 serum, or passively immunized with NP mAb (p). Control mice (○) were previously exposed to PBS (control for H3N2), vaccinated with adjuvant alone (control for rNP), passively immunized with nonimmune serum (control for H3N2 serum), or passively immunized with irrelevant mouse IgG2a mAb (control for NP mAb). Active and passive immunity to NP significantly suppressed levels of mRNA encoding IL-6, CCL2, CXCL1, and platelet-activating factor receptor but not IFN-γ (all p < 0.05 by Student t test versus respective controls).
use of live attenuated influenza vaccines or NP-containing subunit vaccines because classical inactivated influenza vaccines only contain low quantities of NP and weakly boost NP Ab responses (18). Passive immunotherapy using NP-specific Ab also may be useful, particularly for those who respond inadequately to active immunization regimens, such as the immunocompromised and elderly. Finally, by demonstrating that preexisting immunity to influenza NP impacts susceptibility to secondary pneumococcal infection, this report opens new lines of investigation for those studying the pathology, epidemiology, treatment, and prevention of pneumonia, bronchitis, sinusitis, otitis media, and other bacterial diseases commonly associated with influenza infections (1, 3, 6, 51–53).

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References


