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Role of Pneumococcal NanC in the Severe Disease of Streptococcus pneumoniae Superinfection with Influenza

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ABSTRACT

Bacterial superinfection aggravates the disease of influenza. Streptococcus pneumoniae is the most common bacterial pathogen. Synergistic virulence has been demonstrated between influenza neuraminidase and pneumococcal NanA and NanB. NanC, the other pneumococcal neuraminidase infrequently present in clinical isolates, is not well characterized. In this study, we report that superinfection with a NanC-negative pneumococcal strain suppresses anti-influenza immunity and impairs viral clearance with higher TGF-β activation in mice. Bacterial load in the lungs also increases as the host immunity is suppressed. NanC-positive isogenic mutant reverses wild type S. pneumoniae-mediated immune suppression and facilitates virus clearance. However, it causes more severe disease as the augmented inflammation causes collateral damage. Both virus-mediated damage and immune response-mediated inflammation are important for pathogenesis of severe influenza. Inflammation may be more critical than virus-mediated damage in influenza with bacterial superinfection. ImmunoHorizons, 2021, 5: 210–218.

Influenza virus infection is a substantial burden on public health worldwide. Bacterial superinfection aggravates influenza with increased morbidity and mortality. Streptococcus pneumoniae is the most common pathogen for superinfection (1–6). Neuraminidase (NA) is a virulence factor for both influenza virus and S. pneumoniae. There are three pneumococcal NAs: NanA, NanB, and NanC. Synergistic virulence has been shown between NanA and influenza NA. NanB may compensate for the effect of NanA (7). NanC is the least characterized as it is not present in the majority of clinical isolates of S. pneumoniae.

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Abbreviations used in this article: DANA, 2-deoxy-2,3-dihydro-β-N-acetylneuraminic acid; F, forward; HA, hemagglutinin; NA, neuraminidase; PR8, A/PR/8/34; R, reverse; spec, spectinomycin.

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It is present in one third to half of the tested strains (8–11). NanC, just like NanA and NanB as well as influenza NA, cleaves α2,3-linked sialic acids to produce N-acetyllactosaminic acids (9, 12–14). 2-Deoxy-2,3-dihydro-N-acetyllactosaminic acid (DANA) is the N-acetyllactosaminic acid NanC produces. As DANA inhibits NanA and NanB enzymatic activity, it is interesting to learn the role of NanC in influenza with pneumococcal superinfection (10, 15, 16).

The clinical outcome relies on the timing of the two infections in the mouse experimental model. Influenza virus infection with S. pneumoniae superinfection, both in nonlethal doses, on day 3 to day 7 causes 100% lethality. Influenza virus and S. pneumoniae coinfection on the same day causes 50% mortality, and influenza virus infection with a prior exposure of S. pneumoniae 7 d earlier causes no lethality (2). As the lethal synergism requires an influenza virus infection before the challenge with S. pneumoniae (2, 6), influenza virus infection–mediated alterations of host respiratory epithelium and host immunity were the focus of research. However, bacterial factors of the S. pneumoniae may play a role as well through the interactions with influenza virus and host immune response during the superinfection.

In this study, we study the role of pneumococcal NanC on anti-influenza immunity during the superinfection through the use of our influenza hemagglutinin (HA) Ag-specific transgenic mouse model. In our model, influenza virus in the lungs reach its peak by days 2–4, and no plaque forming viable virus can be detected by day 14 if mice survive the infection (17–20). We do adoptive transfer of naive influenza HA-specific CD4^+ T cells at the time of influenza virus infection to study the Ag-specific T cell response to influenza virus infection in vivo. All the HA-specific CD4^+ T cells have all the TCRs responsive to influenza HA only (17–20). They respond to the infection with a Th1 phenotype and help in virus clearance but also contribute to lung inflammation (17, 19). We use this model to study influenza with S. pneumoniae superinfection and the superinfection-mediated alteration of anti-influenza immunity.

**Materials and Methods**

**Ethics statement**

All experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the Chang Gung Memorial Hospital of Taiwan (approval no. 2015092102). The committee recognizes that all the experiments involving the use of mice were carried out in strict accordance with the Animal Protection Law by the Council of Agriculture (Executive Yuan, Taipei City, Taiwan) and the guidelines as shown in the guide for the Care and Use of Laboratory Animals as promulgated by the Institute of Laboratory Animal Resources (National Research Council).

**Transgenic mice**

The TCR transgenic mouse line 6.5, which expresses a TCR recognizing an I-E\(^{d}\)-restricted HA epitope (\(^{110}\)SFERFEIFPKE\(^{120}\); generously provided by H. Von Boehmer, Harvard University, Boston, MA) were BALB/c genetic background. We used mAb against I-E\(^{d}\)-restricted HA-specific TCR (anti-6.5) as a marker for 6.5 CD4^+ T cells. In some experiments, we used Thy-1.1 as a surrogate marker for 6.5 TCR if Thy-1.1 - 6.5 CD4^+ T cells were adoptively transferred into Thy-1.2/1.2 recipients (17–20). Wild type BALB/c mice were purchased from the National Animal Laboratory Center (Taipei City, Taiwan). All mice were maintained in a specific pathogen-free environment and were used for experiments between the ages of 8–24 wk.

**Infection and monitoring**

The A/PR/8/34 (PR8) strain of influenza virus was produced in the allantoic fluid of 10-d-old embryonated chicken eggs and characterized by a core facility at the Chang Gung University. Mice were inoculated intranasally during light isoflurane anesthesia with stated doses of virus in 50 μl PBS (17–20). A clinical isolate of S. pneumoniae serotype 3 (Taian strain, 21) was grown in Todd–Hewitt broth supplemented with 0.5% yeast extract to midlogarithmic phase (OD at 600 nm = 0.2–0.5). The bacterial cultures were then diluted (using PBS) to the desired cell density (CFU/ml) using a previously determined standard curve (OD versus actual CFU) generated for pneumococci. The nominal sizes of the inoculating doses were confirmed by viable counts after plating on blood agar plates. We inserted pneumococcal nanC gene into the Taian bacterial strain as described below and cultured the nanC-inserted bacteria similarly. Mice received superinfection through intranasal inoculation of bacteria in 50 μl PBS on day 4 post-influenza virus infection during light isoflurane anesthesia. Infected mice were monitored every day for disease manifestation. Mice with severe sickness were euthanized humanely in accordance with the guidelines provided by the institutional ethical committee.

**Virus titer determination**

We measured live virus titer in organs of influenza virus–infected mice using a modified Madin Darby canine kidney cell (American Type Culture Collection) plaque assay (17–20). The organs were collected at the indicated times in 1 ml DMEM, snap frozen in liquid nitrogen, and stored at −80°C until analyzed. The 10-fold dilutions of the tissue homogenates were prepared in DMEM supplemented with 10% FCS, antibiotic–antimycotic (15240-062, Life Technologies BRL), and 0.00025% trypsin. A total of 500 μl of each dilution was added to confluent monolayers of Madin Darby canine kidney cells in six-well plates and incubated for 1 h at 35°C with 5% CO\(_2\). Each well of the culture received 2 ml of an agar overlay (0.3%) and was incubated for 3 d. Cells were fixed with 10% formalin, agar overlay was removed, and fixed monolayers were stained by crystal violet (0.02% in...
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mice. Clonotypic percentage was determined by flow cytometry.

Bacterial load determination

At the indicated days, lungs were collected from infected mice. Each lung was kept in 1 ml DMEM. Serial dilutions of the tissue homogenates were plated to blood agar. Bacterial cultures were then diluted using PBS and the number of viable pneumococci was determined using a previously determined standard curve (OD at 600 nm versus actual CFU) generated for pneumococci (21).

Histopathology

The lungs from experimental mice were harvested on the stated days postinfection and fixed with 10% neutral buffered formalin solution. Following fixation, the lungs were embedded in paraffin, and 5-μm sections were cut. Sections were stained with H&E and scored blindly. The infiltration of inflammatory cells, including lymphocytes, neutrophils, and plasma cells, was separately scored by the pathologist as negative, 1+, 2+, or 3+, according to the density of infiltration. Vasculitis and fibrosis were also scored as negative, 1+, 2+, or 3+ based on the severity. Overall inflammation in the lungs was represented by the average of these scores, as described earlier (17–20).

Adoptive transfer

Clonotypic HA-specific CD4+ TCR transgenic T cells were prepared from pooled spleen and lymph nodes of 6.5 transgenic mice. Clonotypic percentage was determined by flow cytometric analysis. The naive phenotype was confirmed by profiles of activation markers CD44 and CD62L were used for surface staining. For intracellular cytokine staining, single-cell suspensions (5–10 million cells/well of a 24-well plate) were restimulated for 5 h with 100 μg/ml MHC class II–restricted HA peptide (H5FERFEIPKKE120) for clonotypic CD4+ T cells in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich). The concentration of brefeldin A was maintained throughout the intracellular cytokine staining. Restimulated cells were surface stained with anti-6.5 and anti-CD4, as described above, fixed in intracellular fixation buffer (eBioscience), washed, and stained in permeabilization buffer (eBioscience) containing fluorochrome-conjugated Abs against target cytokines. Phosphorylated ZAP-70 and phosphorylated STAT1 were stained by following protocols provided by the supplier (BD Biosciences). T-bet was stained ex vivo without restimulation. All fluorochrome-conjugated Abs were from BD Biosciences, except for T-bet and isotype IgG1k (eBioscience). Cells were acquired on FACSCalibur, and analysis was performed using CellQuest Pro (BD Biosciences) or FlowJo (Tree Star) software.

Pneumococcal strain characterization and NanC insertion

Genomic DNA of the S. pneumoniae serotype 3 clinical isolate, Taian (21, 22), was prepared, and NanA, NanB, and NanC genes were PCR amplified using the nanA forward (F) (5'-ATA-GACGTGCGCAAAAATACAGAATC-3') and reverse (R) (5'GGT- CGAACTCCAAGCCAATAACTCTT-3') primers, nanB F (5'-AC- TACGAGGTGTTAATCGTAGG-3') and R (5'-CCCAATACCG- GCAGGCTAAACATC-3') primers, and the nanC F (5'-TGGG-GTAAGTACAAACAGAGG-3') and R (5'-CTATGGTACT- GGCGAAAATAC-3') primers. PCR conditions were 30 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 15 s, and extension at 72°C for 45 s when screening for nanA and nanB. Thirty cycles of denaturation at 96°C for 30 s, annealing at 51°C for 15 s, and extension at 72°C for 45 s were used to screen nanC (22).

The nanC-inserted strain was generated by inserting a single copy of the nanC gene into the noncoding region between the HMPREF0837_12134 and HMPREF0837_12135. The nanC was PCR amplified from another clinical isolate using nanC-F_Pspec-R (5’-GGTACTAATCAAAATAATGAATATAAATATTAAC-3’, nanC-R2) and nanC-C (5’-TTAATTCTTTTTTCAGATCTCTTCA- ATCCTAAATG-3’) primers. A promoter region of spectinomycin (spec) antibiotic cassette was also PCR amplified from pDL278 plasmid using spec-promoter-F (5’-GGAGATCGATTTTTGGCTTCGTG-3’) and Pspec-R_nanC-F (5’-GGTTAATAATTTTTTTTCTATTATTTTAGATACC-3’) primers. Both products were fused using spec-promoter-F and nanC-R2 primers and cloned into pJET 1.2 cloning vector (Thermo Fisher Scientific). The whole spec antibiotic cassette was then PCR amplified from pDL278 plasmid using spec-promoter-F (5’-GAAGATCGATTCTTTGGCTTCGTG-3’) and spec-R (5’-CTACGATTTTTTTTCTATTATTTTAGATACC-3’) primers. The cassette was subcloned into the NcoI site of Psp-nanC::pJET plasmid to generate Psp-nanC-spec::pJET plasmid. The plasmid was PCR amplified using pJET-F (5’-GAAGATCGATTCTTTGGCTTCGTG-3’) and

Flow cytometry and intracellular cytokine assay

Cell suspensions of the lungs were incubated on ice with saturated concentrations of fluorochrome-labeled mAbs in FACS buffer (PBS plus 0.5% BSA and 0.02% NaN3). Donor HA-specific CD4+ T cells were identified using mAbs, as follows: biotin-conjugated anticalonotypic TCR 6.5 (provided by H. Von Boehmer); streptavidin-PE or streptavidin-PerCP; and PerCP- or FITC-conjugated anti-CD4. The anticalonotypic TCR 6.5 Ab was replaced by anti-Thy1.1 (CD90.1) Abs in the case of transferring Thy1.1+ donor cells into Thy1.2+ recipient mice or anti-Thy1.2 (CD90.2) Abs in case of transferring Thy1.2+ donor cells into Thy1.1+ recipient mice. FITC-conjugated CD44 and PE-conjugated CD62L were used for surface staining. For intracellular cytokine staining, single-cell suspensions (5–10 million cells/well of a 24-well plate) were restimulated for 5 h with 100 μg/ml MHC class II–restricted HA peptide (H5FERFEIPKKE120) for clonotypic CD4+ T cells in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich). The concentration of brefeldin A was maintained throughout the intracellular cytokine staining. Restimulated cells were surface stained with anti-6.5 and anti-CD4, as described above, fixed in intracellular fixation buffer (eBioscience), washed, and stained in permeabilization buffer (eBioscience) containing fluorochrome-conjugated Abs against target cytokines. Phosphorylated ZAP-70 and phosphorylated STAT1 were stained by following protocols provided by the supplier (BD Biosciences). T-bet was stained ex vivo without restimulation. All fluorochrome-conjugated Abs were from BD Biosciences, except for T-bet and isotype IgG1k (eBioscience). Cells were acquired on FACSCalibur, and analysis was performed using CellQuest Pro (BD Biosciences) or FlowJo (Tree Star) software.
pJET-R (5’-AAGAACATCGATTCTCCATGCCAG-3’) primers to prepare a Psp-nanC-spec PCR fragment. The fragment was then ligated into the inverse PCR product of p12133-12136::pGEM-T plasmid to generate p12133-Psp-nanC-spec-12136::pGEM-T plasmid. This plasmid was then transformed into the wild type *S. pneumoniae* strain, Taian, and selected with spec (250 mg/L).

**Statistical analyses**

Data were represented as mean ± SD. We used GraphPad Prism version 5 for Student t test analyses. We considered p values <0.05 as significant.

**RESULTS**

*S. pneumoniae* superinfection aggravates the disease of influenza

In our influenza HA Ag-specific transgenic mouse model (17–20), PR8 strain H1N1 influenza virus of 500 PFU causes mild disease in BALB/c mice. Serotype 3 *S. pneumoniae* (21) of 50,000 CFU caused no disease in these mice. However, 500 PFU influenza virus with day 4 superinfection of 50,000 CFU *S. pneumoniae* caused severe disease (Fig. 1A). All mice died by day 10 (Fig. 1B). The viral (Fig. 1C) and bacterial (Fig. 1D) loads in the lungs were higher than those with virus or bacterial infection only. The wild type serotype 3 *S. pneumoniae* isolate has nanA and nanB only (Fig. 2A). We inserted nanC gene (22) into the wild type strain to make an isogenic mutant with all three NA genes (Fig. 2B). Compared with the wild type *S. pneumoniae*, day 4 superinfection with nanC-positive isogenic mutant caused more weight loss and accelerated death (Fig. 3A, 3B).

![FIGURE 1. Aggravated disease of influenza with superinfection of wild type *S. pneumoniae*.](https://doi.org/10.4049/immunohorizons.2100020)  
Naive BALB/c mice were infected with 500 PFU PR8 strain H1N1 influenza virus on day 0 and then superinfected with 50,000 CFU serotype 3 *S. pneumoniae* bacteria on day 4. All mice were monitored for body weight loss (A) and survival (B). Viral (C) and bacterial (D) loads were measured on stated times after viral infection. Control mice received either PR8 influenza virus on day 0 and PBS on day 4 or PBS on day 0 and *S. pneumoniae* bacteria on day 4. Values are mean ± SD, representative of at least three similar experiments (n = 6/group; ***p < 0.0001; two-tailed p values for unpaired t test).

![FIGURE 2. Wild type *S. pneumoniae* has NanA- and NanB-encoding genes only, not the NanC-encoding gene.](https://doi.org/10.4049/immunohorizons.2100020)  
(A) There are NanA- and NanB-, but not NanC-, encoding genes in the genomic DNA of the Taian strain wild type (WT) *S. pneumoniae*. The NanC-encoding gene was present in the nanC-inserted isogenic mutant of the wild type strain. (B) There are NanA and NanB, but not NanC, complementary DNA in the Taian strain wild type (WT) *S. pneumoniae*. NanC complementary DNA was present in the nanC-inserted isogenic mutant of the wild type strain.

![FIGURE 3. Superinfection of nanC-inserted *S. pneumoniae* causes more severe disease compared with wild type *S. pneumoniae*.](https://doi.org/10.4049/immunohorizons.2100020)  
Naive BALB/c mice were infected with 500 PFU PR8 strain H1N1 influenza virus on day 0 and then superinfected with 50,000 CFU wild type or nanC-inserted *S. pneumoniae* bacteria on day 4. All mice were monitored for body weight loss (A) and survival (B). Control mice received influenza virus only, PR8 virus on day 0, and PBS on day 4. Values are mean ± SD, representative of at least three similar experiments (n = 6/group; ***p < 0.0001; two-tailed p values for unpaired t test).
NanA and NanB of *S. pneumoniae* suppressed influenza Ag-specific immunity

We adoptively transferred naive HA-specific CD4^+^ T cells from the 6.5 CD4^+^ TCR transgenic mice into wild type mice. Recipient mice were infected with influenza virus on the same day and superinfected with either wild type or NanC-competent *S. pneumoniae* bacteria on day 4 with control group of mice receiving PBS on day 4. Other control group mice received adoptive transfer on day 0 and single infection on day 4 with influenza virus, wild type *S. pneumoniae*, or NanC-competent *S. pneumoniae*. (A) Membrane desialylation and (B) TGF-β activation of HA-specific CD4^+^ T cells in the lungs on day 6, as described in text. Naive cells are from control mice that received cell transfer but no infection. Histograms are representatives. Values are mean ± SD of the experimental groups, normalized by the values in naive cells (*n* = 6/group). ***p < 0.000, two-tailed p values for unpaired t test).

**FIGURE 4.** NanC attenuated the membrane desialylation and TGF-β activation of influenza Ag-specific CD4^+^ T cells.

Mice received adoptive transfer of naive influenza HA-specific CD4^+^ T cells and influenza virus on the same day. They were superinfected with either wild type or NanC-competent *S. pneumoniae* bacteria on day 4 with control group of mice receiving PBS on day 4. Other control group mice received adoptive transfer on day 0 and single infection on day 4 with influenza virus, wild type *S. pneumoniae*, or NanC-competent *S. pneumoniae*. (A) Membrane desialylation and (B) TGF-β activation of HA-specific CD4^+^ T cells in the lungs on day 6, as described in text. Naive cells are from control mice that received cell transfer but no infection. Histograms are representatives. Values are mean ± SD of the experimental groups, normalized by the values in naive cells (*n* = 6/group). ***p < 0.000, two-tailed p values for unpaired t test).

NanC of *S. pneumoniae* reversed NanA/NanB-mediated suppression of influenza Ag-specific immunity

Contrary to the infection of wild type *S. pneumoniae*, infection of *nanC*-inserted *S. pneumoniae* isogenic mutant strain caused minimal surface desialylation (Fig. 4A, left panel) and no TGF-β activation (Fig. 4B, left panel) of the HA-specific CD4^+^ T cells. Superinfection with the *nanC*-inserted pneumococcal strain also caused only minimal surface desialylation and TGF-β activation, which was even less than influenza virus infection only (Fig. 4, middle and right panels). Consequently, the Th1 suppression was reversed. There were higher levels of activation profile CD44^{high}CD62L^{low} (Fig. 5A), inflammatory Th1 cytokines IFN-γ and TNF-α (Fig. 5B), T-beta induction (Fig. 5C), and phosphorylation of ZAP-70 and STAT1 (Fig. 5D, 5E).

**NanC mediated aggravated lung pathology, despite more comprehensive virus eradication**

NanC reversed the NanA/NanB-mediated suppression of influenza virus–specific host immunity. There was more efficient clearance of the virus. There was less virus in the lungs; even

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the bacterial loads were comparable (Fig. 6A). However, despite much less virus, NanC-competent isogenic *S. pneumoniae* aggravated the lung pathology (Fig. 6B). Compared with focal inflammation with infiltration of neutrophils, plasma cells, and lymphocytes inside the alveolar spaces only caused by wild type *S. pneumoniae*, NanC-competent isogenic strain caused severe diffuse vasculitis (Fig. 6C).

**DISCUSSION**

Our results demonstrate altered host–pathogen interaction with the pneumococcal NanC in *S. pneumoniae* during influenza virus infection with bacterial superinfection. Without NanC, *S. pneumoniae* superinfection attenuates anti-influenza immunity with increased activation of immunosuppressive cytokine

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**FIGURE 5.** NanC augmented activation of influenza Ag-specific CD4⁺ T cells.

Naive influenza HA-specific CD4⁺ T cells were adoptively transferred at the time of influenza virus infection. On day 4, mice were superinfected with wild type or nanC-inserted *S. pneumoniae* bacteria, and HA-specific CD4⁺ T cells in the lungs were analyzed on day 2 after superinfection, as described in the text. Compared with the superinfection of wild type, nanC-inserted *S. pneumoniae* causes expanded CD44⁺CD62L⁻ population (A), more IFN-γ and TNF-α production (B), increased T-bet induction (C), and increased ZAP-70 and STAT1 phosphorylation (D and E) of the HA-specific CD4⁺ T cells in the lungs. Mice with no infection or with influenza virus infection only served as controls. Dot plots and histograms are representatives, and values are mean ± SD, representative of at least three similar experiments (*n* = 6/group, ***p* < 0.0001).
TGF-β in the influenza HA-specific CD4⁺ T cells. Attenuated immunity impairs virus clearance, and mice succumb to infection with high virus burden. Bacterial burden also increases in this immunosuppressive environment. Alveolar deposits and focal infiltration of immune cells around the alveolar spaces in the lungs are associated with their death. In contrast, with pneumococcal NanC, *S. pneumoniae* superinfection intensifies anti-influenza immunity with suppressed TGF-β activation and augmented proinflammatory IFN-γ and TNF-α responses of the HA-specific CD4⁺ T cells. Mice clear the virus more efficiently with the augmented proinflammatory response. Still, they succumb to death even earlier than the death of mice with superinfection of NanC-deficient *S. pneumoniae* bacteria. The superinfection of nanC-inserted *S. pneumoniae* caused severe lung pathology with diffused vasculitis and perivascular cuffing.

The α2-3–linked sialosides are on the epithelium surface of both mouse and human respiratory tract (18, 23). They are the substrates for NAs of both influenza virus (18, 20) and *S. pneumoniae* (10, 13, 14). We observed more membrane desialylation of HA-specific CD4⁺ T cells with the superinfection of NanC-deficient *S. pneumoniae* than that with the infection of influenza virus only. It may then be assumed that the NanC may additionally cleave more sialosides upon superinfection if all three pneumococcal NAs (NanA, NanB, and NanC) are present in *S. pneumoniae*. However, there was less membrane desialylation with the superinfection of NanC-competent than NanC-deficient *S. pneumoniae*. NanC is known to produce DANA from the sialosides, and DANA inhibits the enzymatic activity of pneumococcal NanA and NanB (10). Perhaps DANA-mediated inhibition resulted in less membrane desialylation. NanC may also compete for the same sialoside substrates. The inhibition and/or competition did not compromise the pneumococcal superinfection as the bacterial loads in the lungs were comparable between NanC-deficient wild type and NanC-competent isogenic *S. pneumoniae* strains.

The viral NA cleaves latency-associated peptide from the latent TGF-β to activate the immunosuppressive form of the cytokine, active TGF-β, during influenza virus infection (17, 24). Pneumococcal NanA also activates TGF-β during *S. pneumoniae* infection (25). We found augmented TGF-β activation with the superinfection of nanC-negative wild type *S. pneumoniae*. Active TGF-β is a broad-spectrum immunosuppressive cytokine that inhibits both innate and adaptive immune responses. It controls the innate response by inhibiting NK cells, macrophages, and neutrophils. It also regulates the adaptive response by inhibiting Ag presentation of dendritic cells, activation of T cells, and suppression of effector T cell function (26–28). Such immune suppression acts as a proviral factor during influenza virus infection (29). We also found higher virus burden upon superinfection of nanC-negative wild type *S. pneumoniae* than with influenza virus infection only.

The NanC of *S. pneumoniae* inhibited TGF-β activation and unleashed the IFN-γ and TNF-α responses of HA-specific CD4⁺ T cells. Attenuation of anti-influenza immunity by superinfection with wild type *S. pneumoniae* was reversed, and virus clearance was more efficient in mice with superinfection of NanC-competent *S. pneumoniae*. The IFN-γ response is the first line of host defense in the control of viral infections (30). The response helps in virus clearance, and IFN-γ supplement protects mice from lethal influenza virus infection (31, 32). It is
essential for CTL activity, both in acute and memory responses (33, 34). However, IFN-γ is a proinflammatory cytokine and aggravates lung inflammation in acute influenza as well (17, 35). Cellular infiltration in the lungs decreases with IFN-γ deficiency in IFN-γ knockout mice. Influenza virus infection still gets controlled in these mice with enhanced mucosal immunity and alternative Th2-predominant responses (35–37). Superinfection of NanC-competent *S. pneumoniae* bacteria intensified IFN-γ response of the influenza HA-specific CD4+ T cells in our experiments. The augmented inflammation caused damage and aggravated disease and mortality, even with more comprehensive virus eradication. This observation is in line with a previous report that demonstrated the contribution of anti-influenza IFN-γ response in the aggravated lung pathology of influenza with *S. pneumoniae* superinfection (38). Taken together, both virus-mediated damage and immune response–mediated inflammation are important pathogenic mechanisms for severe influenza. It is well known that management of inflammation may be as important as control of microbial agents in infectious diseases. Moderation of inflammation may be even more important in influenza with superinfection of certain bacteria, such as NanC-competent *S. pneumoniae*.

**DISCLOSURES**

The authors have no financial conflicts of interest.

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