

# Live Attenuated Influenza Vaccine, But Not Pneumococcal Conjugate Vaccine, Protects Against Increased Density and Duration of Pneumococcal Carriage After Influenza Infection in Pneumococcal Colonized Mice

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**Secondary bacterial infections due to *Streptococcus pneumoniae* and *Staphylococcus aureus*, responsible for excess morbidity and mortality during influenza epidemics, are often preceded by excess bacterial density within the upper respiratory tract. Influenza and pneumococcal vaccines reduce secondary infections within the lungs; however, their effects on upper respiratory tract carriage remain unknown. We demonstrate that a live attenuated influenza vaccine significantly reduces pneumococcal growth and duration of carriage during subsequent influenza to levels seen in influenza-naïve controls. No benefit was seen after pneumococcal conjugate vaccine. Our results suggest that live attenuated influenza vaccines may significantly reduce bacterial disease during influenza epidemics.**

**Keywords.** influenza; LAIV; PCV; pneumococcus; carriage; density.

Infection with influenza A virus (IAV) increases susceptibility to pneumonia, bacteremia, sinusitis, and acute otitis media from several bacterial species, including *Streptococcus pneumoniae* and *Staphylococcus aureus* [1, 2]. Bacterial infections are an

important cause of mortality associated with IAV infection in the absence of preexisting comorbidity [3]. Previous investigations by our lab and others have demonstrated that early influenza vaccination can significantly reduce or prevent severe IAV-mediated pneumococcal lower respiratory tract infections and death [4, 5]. Pneumococcal conjugate vaccine (PCV) has also demonstrated significant efficacy to reduce severe postinfluenza pneumococcal disease [6].

Bacterial lower respiratory tract infections and invasive disease (irrespective of influenza status) often are preceded by a primary colonizing event with dissemination of bacteria to the lower respiratory tract. Further, the probability of developing a lower respiratory tract infection is believed to be associated with increased pneumococcal colonizing density [7].

Animal models and human studies suggest that intranasal infection with IAV can enhance carriage density of pneumococci in the nasopharynx (NP) [1], which likely contributes to the excess incidence of severe pneumococcal disease after IAV infection [8]. Although the mechanisms underlying this excess colonization within the NP are not entirely defined, they include a combination of IAV-mediated cytotoxic breakdown of mucosal and epithelial barriers [9] and aberrant innate immune responses to bacterial invaders in the upper respiratory tract (URT) in the postinfluenza state [1].

We sought to determine the efficacy of the 7-valent PCV and a live attenuated influenza vaccine (LAIV) to prevent excess pneumococcal colonization density and to decrease duration of pneumococcal carriage after infection with IAV.

## METHODS

### Infectious Agents

Viral infections were carried out with an H3N2 reassortant virus (HK/Syd) developed as described previously [10], containing the surface glycoprotein hemagglutinin from A/Hong Kong/1/68 and the neuraminidase from A/Sydney/5/97 (Syd97). The 6 internal protein gene segments are from the St Jude variant of the mouse-adapted influenza virus strain A/Puerto Rico/8/34 (PR8). LAIV vaccinations used a temperature sensitive attenuated variant of HK/Syd (HK/Syds or LAIV) containing the same site-specific mutations as those in the attenuated A/Ann Arbor/6/60 master donor strain used to produce the influenza A virus strains found in the human FluMist vaccine, as described previously [10]. The growth dynamics, safety, efficacy, and antibody response of our LAIV vaccine have been previously described [4].

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All viruses were propagated in 10-day-old embryonated chicken eggs at 37°C and 33°C for the wild-type (WT) and LAIV strains, respectively. The dose infectious for 50% of tissue culture wells (TCID<sub>50</sub>) was performed using Madin-Darby canine kidney cells. The serotype 19F (ST<sup>425</sup>) pneumococcal clinical isolate BHN97 was engineered to express luciferase and used for all experiments because of its colonizing potential, as previously described [2]. The pneumococcal conjugate vaccine (PCV7; Prevenar, Wyeth Vaccines) consisted of capsular polysaccharides conjugated to CRM197 carrier protein of pneumococcal serotypes 4, 6B, 9 V, 14, 18C, 19F, and 23F.

### Animal and Infection Models

Four-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used for all experiments, and all procedures were approved by the Animal Care and Use Committee at St Jude Children's Research Hospital. Mice were vaccinated with PCV7, LAIV, or sham vaccine (phosphate-buffered saline [PBS] vehicle) and monitored for 4 weeks. At 8 weeks, PCV7 recipients received a PCV7 booster. Inoculations were through the intranasal route under general anesthesia with inhaled isoflurane (2.5%), except for PCV7 which was given intramuscular in 0.05 mL. LAIV vaccination consisted of 2e6 TCID<sub>50</sub> HK/Sydney<sub>ts,att</sub> LAIV in 40 µL PBS. Sublethal doses of WT HK/Sydney used 1e5 TCID<sub>50</sub> in 50 µL PBS. Pneumococcal infection consisted of 1e5 colony-forming units in 40 µL of PBS administered intranasally evenly between the 2 nares. For duration of carriage studies, once bacterial density fell below the limit of detection for in vivo imaging (approximately 1e4 colony-forming units [CFUs]), bacterial CFU titers were measured in nasal washes using 12 µL of PBS administered and retrieved from each nare and quantitated by serial dilution plating on blood agar plates (lower limit of detection was 100 CFU/mL nasal wash).

### Bioluminescent Imaging

Mice were imaged using an In Vivo Imaging System (IVIS) CCD camera (PerkinElmer, Waltham, MA) as described [2]. Nasopharyngeal bacterial density was assessed by measuring the flux of light as total photons per second per centimeter squared in prespecified regions covering the NP, and background (calculated for each mouse on a region of equal area over the hind limb) was subtracted. Each NP measurement represents an average of 2 pictures, 1 for each side of the mouse head. Quantitation was performed using LivingImage software (version 3.0; PerkinElmer) as described [2].

### Statistical Analyses

All statistical analyses were performed using the R statistical environment (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>). Pairwise comparisons were

performed using Student *t* tests with Bonferroni correction for multiple comparisons.

## RESULTS

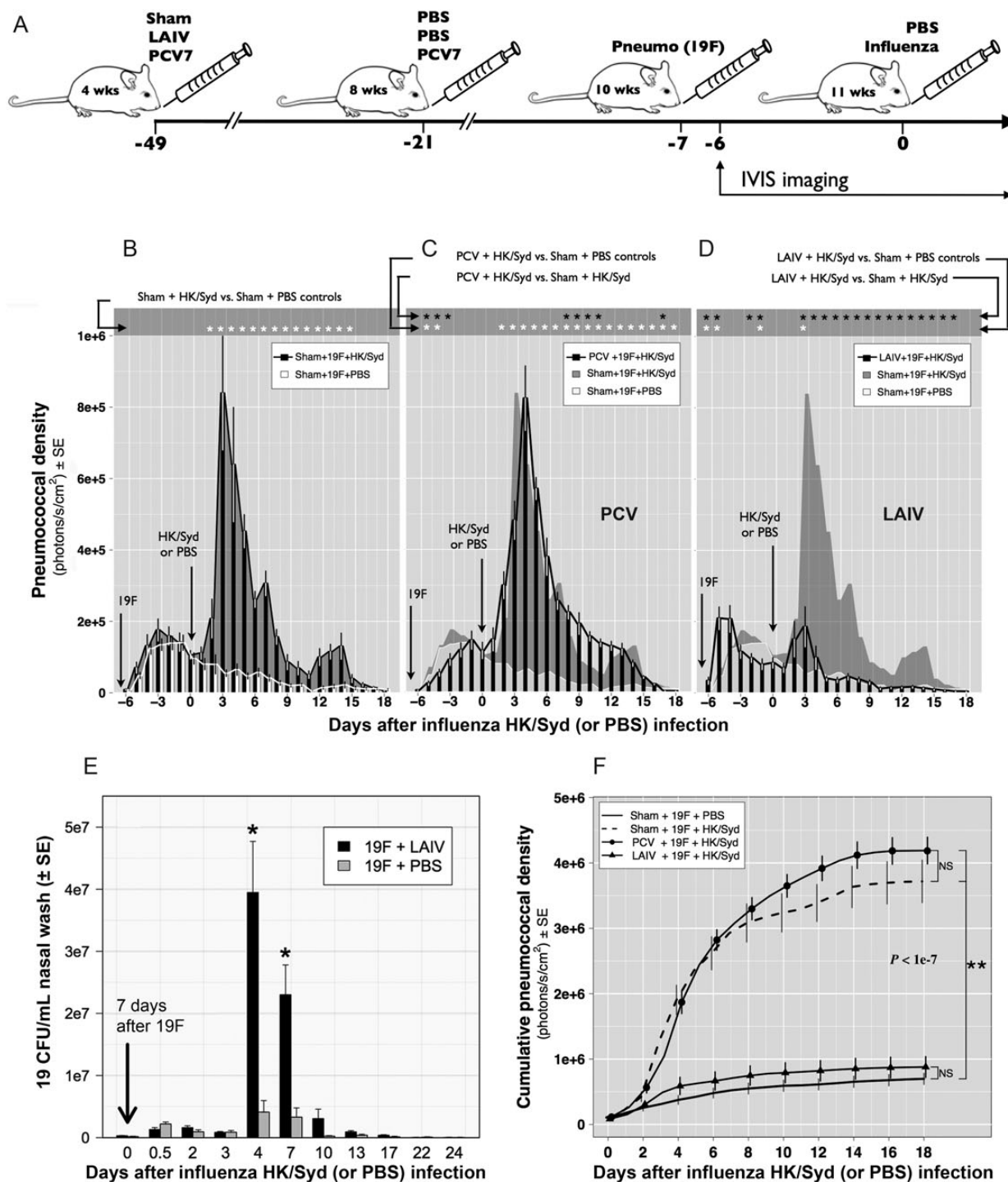
### WT Influenza Virus Enhances Density of Bacterial Colonization in the Nasopharynx

We sought to measure the effect of an H3N2 influenza virus on density of pneumococcal colonization in mice precolonized with 19F pneumococci and without any previous exposure to prophylactic vaccination. Mice were given sham vaccine and 4 weeks later colonized with pneumococci. Seven days after colonization, during normal bacterial clearance, mice were infected with either WT influenza virus or PBS control (referred to hereafter as "controls" [see Figure 1A for a schematic of the experimental design]). Within 12 hours after viral inoculation, normal pneumococcal clearance ceased, and swift, exponential bacterial growth ensued within the NP (Figure 1B). By 2 days after infection with influenza, bacterial density in the NP was significantly increased over controls and maximal bacterial titers were 15-fold higher than PBS controls (8.4e5 vs 5.5e4; *P* < .001). Density remained significantly elevated until 15 days after influenza infection. Overall, cumulative bacterial load was increased nearly 500% as a result of influenza infection (Figure 1F; *P* = 1e–7).

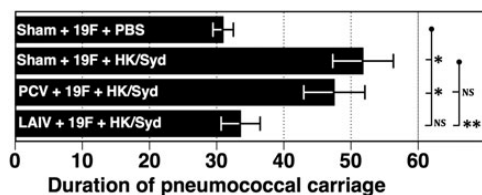
To ensure that the density and dynamics of pneumococcal growth measured by luminescence (Figure 1B–D, F) accurately represents true bacterial growth dynamics within the nasopharynx, the experiment just described was repeated, and nasal washes were performed on select days after influenza infection to quantitate by serial dilution plating the true bacterial CFUs within the NP of influenza-infected vs control mice (Figure 1E). A comparison of Figure 1B and 1E demonstrates agreement between these 2 methods of bacterial quantitation.

### Early Prophylactic LAIV but Not PCV Reduces Excess Pneumococcal Density in the NP After WT Influenza

The effectiveness of early prophylactic LAIV or PCV vaccination to reduce influenza-associated excess bacterial carriage density and duration was assessed. Four-week-old mice received either LAIV, PCV, or sham vaccine, and 4 weeks later, PCV recipients received PCV boosters, whereas LAIV and sham vaccinated mice received PBS. Two weeks after PCV boosters, all mice were colonized with pneumococci, followed 7 days later by intranasal infection with WT influenza virus. Prophylactic vaccination of colonized mice with PCV demonstrated no efficacy to prevent or reduce excess bacterial titers after influenza infection (Figure 1C and 1E) relative to controls. In fact, the dynamics of pneumococcal growth after influenza infection were nearly identical between the PCV and sham-vaccinated groups except that peak density in the PCV group occurred 1 day later than in the sham-vaccinated group.



**Figure 1.** Dynamics of pneumococcal carriage after influenza infection with and without prophylactic vaccination. *A*, Overall experimental design. Groups of 12–15 mice were given either sham vaccine (*B*), pneumococcal conjugate vaccine (PCV) (*C*), or live attenuated influenza vaccine (LAIV) (*D*). Four weeks later all mice were colonized with a type 19F pneumococcus 7 days before intranasal infection with an H3N2 influenza virus (HK/Syd; black bars/dark grey shaded regions) or phosphate-buffered saline (PBS) vehicle control (white bars/white shaded regions). Nasopharyngeal carriage density is plotted (*E*) for days 1 through 24 after 19F colonization (6 days before to 18 days after influenza/PBS inoculation). Density of colonization after PCV and LAIV are plotted (*F*) over grey and white shaded regions representing pneumococcal densities in sham vaccinated and influenza infected or PBS vehicle controls, respectively. To vary the utility of In Vivo Imaging System imaging for bacterial quantification, the experimental design for (*B*) was repeated, and nasal washes were serially diluted and plated on blood agar plates for manual quantitation of bacterial colony forming units (*E*). Cumulative bacterial titers after influenza infection are plotted in (*F*) for each of the 4 groups. Asterisks (\*) indicate significant differences in bacterial titers between respective groups as labeled (Student *t* test with Welch approximation of degrees of freedom; *P* < .05 unless otherwise indicated). Abbreviations: LAIV, live attenuated influenza vaccine; PBS, phosphate-buffered saline; PCV, pneumococcal conjugate vaccine.



**Figure 2.** Duration of pneumococcal carriage. Duration of pneumococcal carriage was measured by daily nasal washes and serial dilution plating beginning after nasopharyngeal titers fell below the limit of detection for in vivo imaging. Interior-most asterisks (\*) indicate significant differences in duration of carriage relative to sham-vaccinated and phosphate-buffered saline-infected controls. Outer asterisks (\*\*) indicate significant differences relative to sham-vaccinated and influenza-infected mice (Student *t* test with Bonferroni correction;  $P < .005$  for all asterisks; NS indicates  $P > .40$ ). Abbreviations: LAIV, live attenuated influenza vaccine; NS, not significant; PBS, phosphate-buffered saline; PCV, pneumococcal conjugate vaccine.

It is interesting to note, however, that in the days just after 19F colonization, before infection with influenza, prophylactic PCV vaccination resulted in 2- to 3-fold decreased pneumococcal titers relative to that in the sham-vaccinated groups ( $P < .05$ ; Figure 1C and 1D). However, any potential benefit initially conferred by early vaccination with PCV was lost entirely after influenza infection.

In contrast, prophylactic LAIV led to significantly reduced excess bacterial outgrowth after WT influenza virus infection (Figure 1D), with maximum excess pneumococcal density after influenza infection only 2-fold increased over PBS controls (compared with the 15-fold increases seen in the sham- and PCV-vaccinated groups), and this increase was significant at only a single time point (3 days after infection with influenza). Early LAIV vaccination also reduced the duration that bacterial titers were elevated over PBS controls 5-fold, to only 3 days of excess titers in the LAIV recipients (vs 15 days in the PCV and sham vaccine groups). Overall, prophylactic LAIV led to a 77% reduction in cumulative pneumococcal titers after influenza inoculation ( $8.8e5$  vs  $3.7e6$ ;  $P = 1.3e-7$ ; Figure 1E), resulting in cumulative titers no different than the noninfluenza-infected PBS controls ( $8.8e5$  vs  $7.0e5$ ;  $P = .33$ ; Figure 1E). In contrast, the PCV-treated group had mean cumulative titers approximately 600% greater than PBS controls ( $4.2e6$  vs  $7.0e5$ ;  $P = 1e-10$ ; Figure 1E).

#### WT Influenza Infection and Prophylactic Vaccination Significantly Alter Duration of Pneumococcal Carriage

Duration of carriage was assessed by daily nasal washes after bacterial NP titers fell below the limit of detection for IVIS. In accordance with human carriage studies, carriage was significantly increased after influenza infection, with bacterial CFUs present in nasal wash for a mean of 52 days after pneumococcal

colonization, vs 31 days in the PBS controls ( $P = .0004$ ; Figure 2). Prophylactic vaccination with PCV had no effect on prolonged duration of carriage (47.5 days vs 52 days;  $P = .51$ ; Figure 2). Early vaccination with LAIV, however, almost entirely abrogated prolonged duration of carriage due to influenza infection with carriage lasting a modest 33.5 days (vs 52 days;  $P = .002$ ; Figure 2). This duration was no different than the mean duration of carriage detected in the influenza-uninfected controls (33.5 days vs 31 days;  $P = .44$ ; Figure 2).

## DISCUSSION

Although vaccine efficacy is traditionally measured as the ability to directly prevent disease from the vaccine target pathogen, it is important to consider indirect benefits of vaccination as well. In the context of influenza vaccination, an important indirect benefit is prevention of secondary bacterial disease.

Influenza infections drive pneumococcal transmission and disease. In agreement with studies in humans [11], we have shown here, in mice, that H3N2 influenza virus significantly enhances pneumococcal density and duration of carriage within the NP. Excess bacterial density in the URT enhances risk of lower [7] and upper respiratory infections, such as acute otitis media [12], in humans and has been shown to increase the likelihood of transmission [13]. Extended duration of carriage further increases opportunity for transmission.

Pneumococcal conjugate vaccine has been extremely successful in reducing overall incidence of invasive pneumococcal disease [14] and has shown efficacy to reduce severe influenza–pneumococcal coinfections of the lower respiratory tract within vaccinated individuals [6]. However, key to efficient and wide-scale prevention of excess bacterial coinfections during influenza epidemics is disruption of influenza-induced transmission events across the general population [15]. We have demonstrated here a desirable secondary effect of influenza vaccination to significantly reduce influenza-mediated excess pneumococcal carriage density, an important contributor to pneumococcal transmission [13]. That this effect was not achieved through the use of pneumococcal conjugate vaccine is in keeping with the observation that PCVs reduce pneumococcal serotype specific carriage by reduction in acquisition [14] but have not been shown to reduce density of existing carriage or duration of carriage. Although PCVs may reduce lower respiratory infections of vaccinated hosts who become coinfecting with influenza and pneumococcus, our results suggest benefit normally conferred by PCV to prevent upper respiratory tract colonization and subsequent transmission may be compromised in the setting of an influenza infection. Meanwhile, LAIV demonstrated significant efficacy in reducing postinfluenza pneumococcal carriage density and duration to levels near those seen in the absence of influenza infections.



Our data suggest that effective LAIV immunization may have a greater impact on influenza-associated pneumococcal transmission than concurrent PCV administration, emphasizing the importance of prophylactic rather than reactive PCV immunization to prevent pneumococcal transmission during influenza infection.

## Notes

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**Potential conflict of interest.** K. P. K. has received consulting fees from Pfizer and G.S.K., manufacturers of pneumococcal conjugate vaccines, and research funding for studies unrelated to this study from Pfizer. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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