Live Attenuated Influenza Vaccine Enhances Colonization of *Streptococcus pneumoniae* and *Staphylococcus aureus* in Mice

Michael J. Mina,[a,b,c] Jonathan A. McCullers,[c,d] Keith P. Klugman[b]

Medical Scientist Training Program, Emory University School of Medicine, Atlanta, Georgia, USA[a]; Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA[b]; Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, USA[c]; Department of Pediatrics, University of Tennessee Health Sciences Center, Memphis, Tennessee, USA[d]

ABSTRACT **Community interactions at mucosal surfaces between viruses, like influenza virus, and respiratory bacterial pathogens are important contributors toward pathogenesis of bacterial disease. What has not been considered is the natural extension of these interactions to live attenuated immunizations, and in particular, live attenuated influenza vaccines (LAIVs). Using a mouse-adapted LAIV against influenza A (H3N2) virus carrying the same mutations as the human FluMist vaccine, we find that LAIV vaccination reverses normal bacterial clearance from the nasopharynx and significantly increases bacterial carriage densities of the clinically important bacterial pathogens *Streptococcus pneumoniae* (serotypes 19F and 7F) and *Staphylococcus aureus* (strains Newman and Wright) within the upper respiratory tract of mice. Vaccination with LAIV also resulted in 2- to 5-fold increases in mean durations of bacterial carriage. Furthermore, we show that the increases in carriage density and duration were nearly identical in all aspects to changes in bacterial colonizing dynamics following infection with wild-type (WT) influenza virus. Importantly, LAIV, unlike WT influenza viruses, had no effect on severe bacterial disease or mortality within the lower respiratory tract. Our findings are, to the best of our knowledge, the first to demonstrate that vaccination with a live attenuated viral vaccine can directly modulate colonizing dynamics of important and unrelated human bacterial pathogens, and does so in a manner highly analogous to that seen following wild-type virus infection.**

IMPORTANCE **Following infection with an influenza virus, infected or recently recovered individuals become transiently susceptible to excess bacterial infections, particularly *Streptococcus pneumoniae* and *Staphylococcus aureus*. Indeed, in the absence of preexisting comorbidities, bacterial infections are a leading cause of severe disease during influenza epidemics. While this synergy has been known and is well studied, what has not been explored is the natural extension of these interactions to live attenuated influenza vaccines (LAIVs). Here we show, in mice, that vaccination with LAIV primes the upper respiratory tract for increased bacterial growth and persistence of bacterial carriage, in a manner nearly identical to that seen following wild-type influenza virus infections. Importantly, LAIV, unlike wild-type virus, did not increase severe bacterial disease of the lower respiratory tract. These findings may have consequences for individual bacterial disease processes within the upper respiratory tract, as well as bacterial transmission dynamics within LAIV-vaccinated populations**

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Address correspondence to Michael J. Mina, mmina@emory.edu.

The conventional view of pathogen dynamics posits that pathogen species act independently of one another. More recently, however, community interactions between pathogens have been recognized as necessary to modulate both health and disease (1–7). These interactions might be expected to be most prevalent within gut, respiratory, and other mucosal surfaces that harbor complex populations of commensal and, occasionally, pathogenic microbes. In the respiratory tract, for example, viral infections are known to predispose to secondary bacterial invasive disease and pneumonia from pathogens that are most commonly benign but occasionally become virulent, particularly following a viral infection (8–10). A well-known example is the often lethal synergy between influenza virus and pneumococcal or staphylococcal bacterial secondary infections.

Infection with influenza viruses increases susceptibility to severe lower and upper respiratory tract (LRT and URT, respectively) bacterial infections resulting in complications, such as pneumonia, bacteremia, sinusitis, and acute otitis media (11). Bacterial infections may be a primary cause of mortality associated with influenza virus infection in the absence of preexisting comorbidity (12, 13). Primary influenza virus infection increases acquisition, colonization, and transmission of bacterial pathogens (14), most notably the pneumococcus *Streptococcus pneumoniae* and *Staphylococcus aureus* (11, 15).

Although the underlying mechanisms, while well studied, are not entirely defined, they likely include a combination of influenza virus-mediated cytotoxic breakdown of mucosal and epithelial barriers (16–18) and aberrant innate immune responses to

bacterial invaders in the immediate postinfluenza state, characterized by uncontrolled pro- and anti-inflammatory cytokine production, excessive leukocyte recruitment, and extensive immunopathology (11, 19–22). When coupled with diminished epithelial and mucosal defenses, such an environment becomes increasingly hospitable for bacterial pathogens to flourish and invade in the days and first few weeks following influenza virus infection.

Increasingly, evidence is linking the early innate immune response triggered by infection or vaccination to sustained adaptive immunity (23). Thus, a broad goal of vaccination is to elicit an immune response analogous to that of the pathogen itself, without subsequent disease (24). The intranasally administered live attenuated influenza vaccine (LAIV) contains temperature-sensitive and attenuated virus designed to replicate efficiently in the cooler temperatures of the upper respiratory tract (URT) but which fails to do so in the warmer temperatures of the lower respiratory tract (LRT) (25, 26). Through selective replication in the URT, LAIV proteins are exposed to the host immune system in their native conformation, eliciting highly robust (IgA), serum (IgG), and cellular immune responses mimicking those of the pathogenic virus itself (27).

Although an innate immune response to vaccination is beneficial for long-term protection from influenza virus (28) and influenza virus-bacterial (29) coinfections, the direct consequences of such a response to a viral vaccine, with respect to secondary colonization and disease due to entirely unrelated bacterial pathogen species, are unknown. As increased susceptibility to and transmission of bacterial pathogens following influenza are due in large part to the innate immune response and breakdowns of the epithelial barriers of the URT, it is important to understand whether similar effects, elicited by live attenuated virus replication, may also predispose to bacterial infection. We sought here to determine the effects of a live attenuated influenza vaccine on URT and LRT bacterial infections. In particular, we ask whether LAIV vaccination alters bacterial colonization dynamics of the upper respiratory tract or disease in the lower respiratory tract of mice.

RESULTS

Using a live attenuated influenza A virus vaccine, HK/Syd 6:1:1 (LAIV), which contains many of the same mutations and demonstrates similar growth dynamics to those in the commercially available human FluMist vaccine (MedImmune, Gaithersburg, MD) (see reference 30 and Fig. S1 in the supplemental material for vaccine details), we evaluated the effects of LAIV and its wild-type (WT) HK/Syd parent strain (referred to as WT virus) on *Streptococcus pneumoniae* (the pneumococcus) and *Staphylococcus aureus* replication and disease.

LAIV virus is restricted in growth at 37°C but not at 33°C. To determine whether LAIV virus grows efficiently at temperatures seen within the nasopharynx (NP) while remaining restricted in growth at warmer temperatures of the LRT, WT influenza virus and its LAIV derivative were grown in MDCK cells at 37°C. As expected (30), a >3-log decrease in viral titers was measured for LAIV relative to the WT parent strain ($P < 0.001$) (Fig. 1A). However, when LAIV was propagated at 33°C, a temperature often associated with the nasopharyngeal environment (31), viral replication was no different from that of WT virus titers measured at 37°C.

HK/Syd 1:1:6 LAIV vaccination is safe and effective in mice. Although LAIV is attenuated, inoculation with very high doses may cause morbidity and weight loss. Via a series of dosing experiments (data not shown), a vaccinating dose of 2e6 tissue culture infective doses ($TCID_{50}$) of LAIV in 40 μl phosphate-buffered saline (PBS) vehicle was determined to be safe, with no weight loss or other detectable signs of morbidity in mice (Fig. 1B). This dose is in agreement with previous studies (28, 30). Inoculation with the same dose of the WT parent virus led to significant morbidity and mortality (5/12 mice succumbed by day 7 postinfection) (Fig. 1B), demonstrating the attenuated nature of the LAIV.

The vaccine efficacy and antibody response using this LAIV strain were described previously (30). To phenotypically confirm efficacy here, groups of 8 4-week-old mice were inoculated with LAIV or the PBS control and 4 weeks later with a lethal dose of the WT virus. Early vaccination with LAIV conferred complete protection from any detectable morbidity or weight loss due to infection with the WT strain, versus 100% mortality in unvaccinated control mice (Fig. 1C).

LAIV is restricted in growth in the lower but not the upper respiratory tract. To determine whether the differences in replication seen *in vitro* also occur *in vivo* in the upper (~33°C) versus lower (~37°C) respiratory tract, groups of 5 mice were vaccinated with LAIV, and viral titers were measured in whole lung and whole NP homogenates (Fig. 1D). By 3 days postvaccination, NP titers were 10,000-fold greater than in the lungs (1.3e6 versus 1.2e2 $TCID_{50}$; $P < 0.001$). In contrast, the WT virus grew to high viral titers in both the NP and lungs (>5e5 $TCID_{50}$) (data not shown), in agreement with previous reports (32), which led to significant morbidity and mortality, as demonstrated in the controls in Fig. 1B. Overall, maximal NP titers occurred earlier and were nearly 400-fold greater than maximum lung titers (1.3e6 versus 3.4e3 $TCID_{50}$; $P < 0.001$). Importantly, these NP viral dynamics are in agreement with viral shedding in NP aspirates from human subjects following vaccination with the FluMist vaccine (33).

LAIV cytokine response in the nasopharynx and lungs. While LAIV replication in the NP induces a robust systemic inflammatory response (34, 35), the cytokine response in the NP has, to our knowledge, not been observed. Nasopharyngeal homogenates and bronchoalveolar lavage (BAL) specimen cytokines were measured in groups of 5 mice each at days 0, 3, 5, and 7 postvaccination (Fig. 1E). Of particular interest, the type I interferon (IFN-β) was significantly increased in the NP and BAL specimens following LAIV vaccination, and this cytokine has been demonstrated to play a pivotal role in excess bacterial colonization of the nasopharynx following WT influenza virus infection (36). As well, macrophage inflammatory protein 1β (MIP-1β) was also significantly upregulated following LAIV, similar to what was seen following influenza virus-pneumococcal coinfections of human middle ear epithelial cells (37). In general, the responses measured here in the NP are similar to those measured from nasopharyngeal washes in humans infected naturally with seasonal influenza A viruses (38).

LAIV enhances pneumococcal bacterial dynamics in the URT in a manner highly analogous to WT influenza virus. Numerous previous investigations have demonstrated that replication of WT influenza virus within the URT predisposes to excess bacterial replication and colonization within the NP, particularly by *Streptococcus pneumoniae* (36, 39, 40). Because, as demonstrated above, LAIV replicates to near WT levels when in the cooler temperatures of the URT, we sought to study effects of LAIV on bacterial carriage density within the NP of mice and compared them to the changes in bacterial carriage following WT

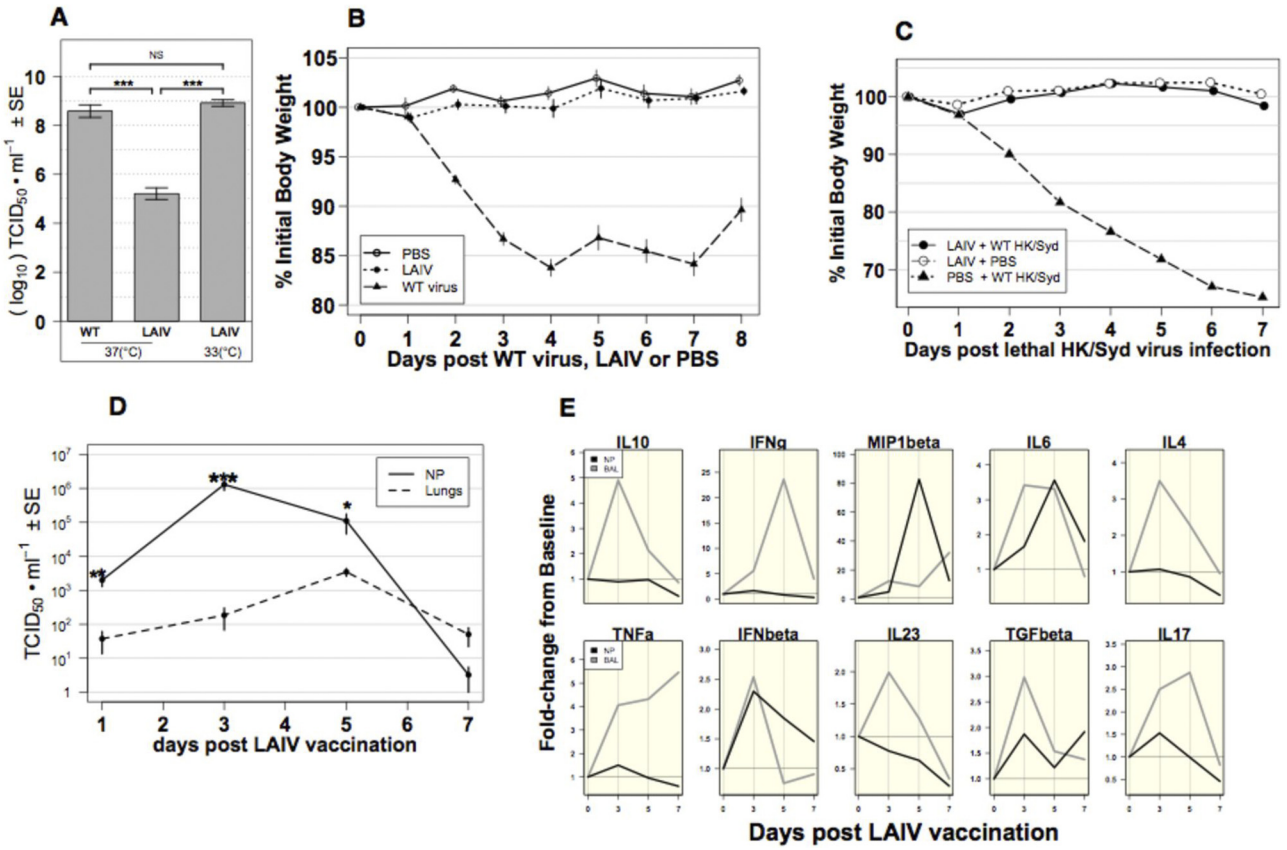

FIG 1 LAIV is safe, effective, replicates well within the URT, and elicits a robust cytokine response. (A) WT and LAIV HK/Syd viruses were grown in MDCK cells at 37°C and LAIV virus was grown at 33°C, and viral titers were measured via the median TCID50 (n = 3 per group). (B) Groups of 12 to 14 8-week-old BALB/c mice were inoculated with 2e6 TCID50 LAIV, WT HK/Syd virus, or PBS and monitored for weight loss. Three of 12 mice and 2/12 mice died at 4 and 7 days postinfection with WT HK/Syd virus, respectively, while no mice died following LAIV or PBS inoculation. (C) Groups of 8 4-week-old BALB/c mice were inoculated with 2e6 TCID50 of LAIV (2 of the 3 groups) or PBS and 4 weeks later infected with a lethal dose (5e7 TCID50) of WT HK/Syd virus or the PBS control. Infection was considered lethal if body weight fell below 70% of the initial body weight. (D) Four groups of 5 mice each were vaccinated with LAIV, and whole lung and NP viral titers were measured at 1, 3, 5, and 7 days postvaccination. (E) Four groups of 5 mice were vaccinated with LAIV, and NP and BAL specimen cytokines were measured at day 0 (unvaccinated mice) and days 3, 5, and 7 following vaccination. Error bars represent standard errors (SE) of the mean. Asterisks indicate statistically significant differences from controls by two-sided Student's t test. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. NS, not significant (no difference between groups).

virus infection. LAIV vaccination or sublethal infection with the WT parent strain was delivered 7 days following inoculation with a common nasopharynx-colonizing strain of pneumococcus type 19F (Fig. 2A to C) included in the current pneumococcal conjugate vaccine (41). Following vaccination, normal bacterial clearance from the NP was halted, and bacteria reverted to exponential growth within 3 days postvaccination (Fig. 2B). Receipt of LAIV significantly increased the density of bacterial carriage and extended the mean duration of colonization from 35 to 57 days (Fig. 2C). Of particular importance, these effects were nearly identical in all aspects to the effects of the WT influenza virus on bacterial carriage density and duration (Fig. 2B and C). Although no detectable morbidity was associated with vaccination alone (Fig. 1B), vaccination in the presence of bacterial colonization resulted in very mild, though sustained weight loss (~3 to 5%; $P <$ 0.05) relative to colonized, unvaccinated controls (see Fig. S2 in the supplemental material) that corresponded with time of greatest excess bacterial proliferation.

To test whether order and timing of vaccination relative to bacterial acquisition are important, LAIV or WT virus was administered 7 days before (rather than after) 19F colonization (Fig. 2D to F). Early vaccination or infection with WT virus led to immediate excess bacterial outgrowth following pneumococcal inoculation relative to that in mice pretreated with PBS vehicle (Fig. 2E). This increase was generally more pronounced following LAIV vaccination relative to WT virus infection, but the difference only reached statistical significance at day 1 post-bacterial infection. Increases in mean durations of carriage were also demonstrated and were similar between the two groups, with duration extending from 38 days following treatment with PBS to 63 or 65 days following LAIV or WT virus infection, respectively (Fig. 2F).

To further define the temporal nature of these interactions and simultaneously test whether this response is strain specific, vaccination was given at either 1 or 7 days prior to infection with a slightly more invasive type 7F pneumococcus (Fig. 3A). The maximum bacterial density in both groups of vaccinated mice reached a near 100-fold increase versus that in PBS controls. When inoculation with bacteria followed only 1 day (versus 7 days) postvaccination, similar but delayed dynamics (Fig. 3A) and cumulative bacterial titers (Fig. 3B) were measured. Interestingly, the delay

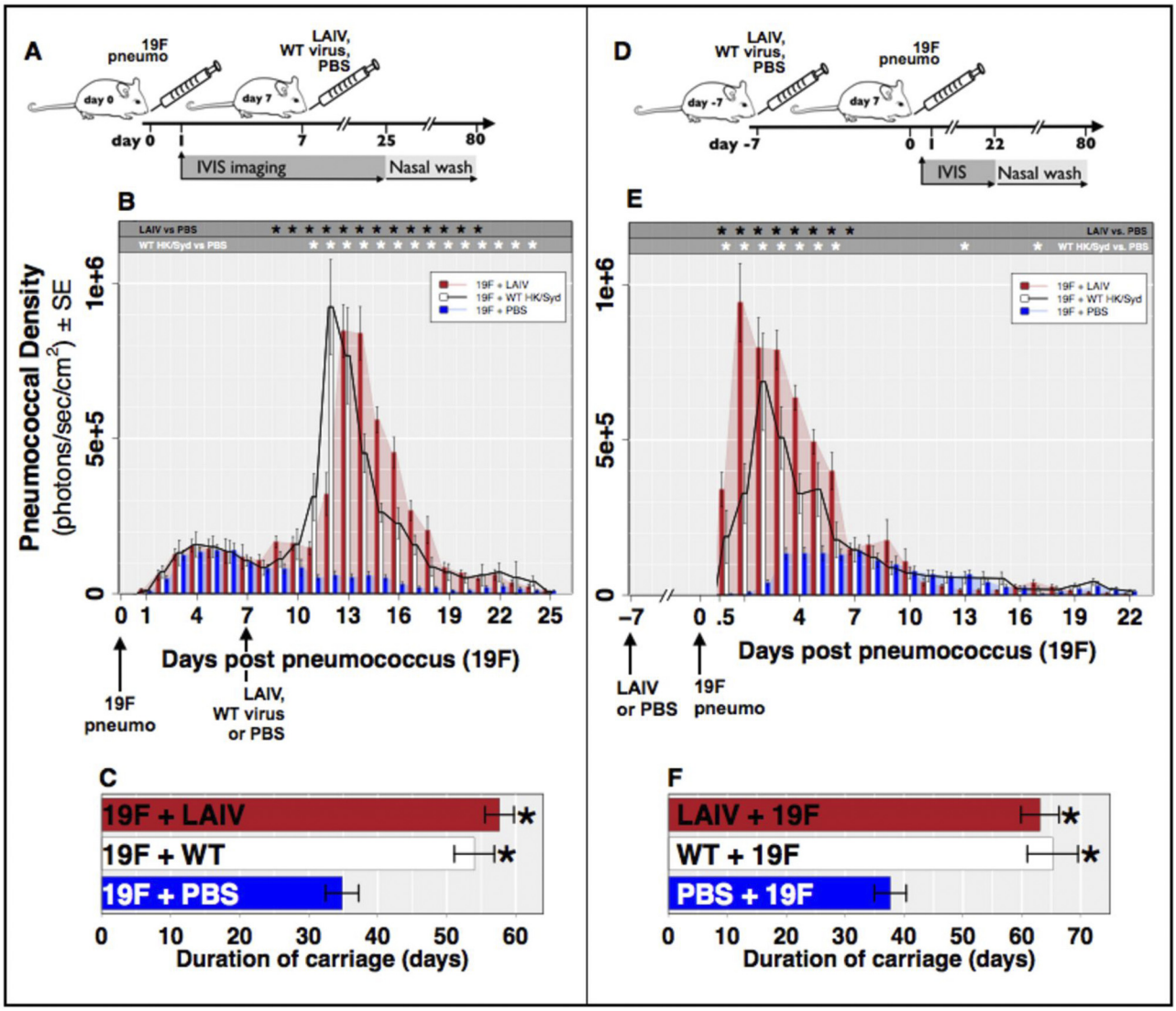

FIG 2 LAIV and WT influenza virus infection similarly enhance 19F pneumococcal carriage density and duration of colonization. Groups of 12 to 14 mice were vaccinated with LAIV and infected with WT influenza virus or PBS vehicle at 7 days following colonization with 19F pneumococcus (A to C) or 7 days prior to colonization with 19F (D to F). Bacterial strains constitutively expressed luciferase, and nasopharyngeal carriage density was measured via in vivo imaging (IVIS) at 12 h postbacterial infection and daily thereafter (B and E). Duration of colonization (C and F) was measured via bacterial plating of nasal washes taken daily after carriage density decreased below the limit of detection for IVIS imaging (~1e4 CFU/ml). Asterisks indicate significant differences between vaccinated (black asterisks in panels B and E) or WT influenza virus-infected (white asterisks in panels B and E) versus control groups ($P < 0.05$ by Students t test), and error bars represent standard errors around the mean.

was consistent with the difference in times from vaccination to bacterial inoculation between the two groups.

We sought to understand whether these effects of LAIV vaccination on bacterial proliferation would continue over a longer duration. Mice were infected with pneumococcus 28 days following LAIV vaccination—well after viral clearance from the NP was complete (~7 days postvaccination). Despite the 28-day lag between LAIV and pneumococcal infection, LAIV continued to yield immediate excess bacterial proliferation relative to PBS controls (Fig. 3C); however, the effect was modest and short-lived, with only 2- to 4-fold increases over PBS controls measured between days 1 and 3 postinfection, respectively. By day 4, bacterial density in the NP returned to control levels, and the duration of colonization was not increased.

LAIV enhances *Staphylococcus aureus* dynamics in the URT. We next sought to test the effects of LAIV on carriage of an entirely distinct but important Gram-positive bacterium, *Staphylococcus aureus*. LAIV was administered 7 days prior to infection with *S. aureus* strain Wright (Fig. 4A and B) or Newman (Fig. 4C and D). Similar to the previous experiments using two strains of pneumococcus, the density of these two strains of *S. aureus* following vaccination was increased at all measured time points for both the Wright and Newman strains (Fig. 4A and C), and duration of colonization was significantly extended 3- to 5-fold over that in the PBS controls (Fig. 4B and D).

LAIV does not increase morbidity or mortality from bacterial LRT infections. Given the severe and often lethal interaction seen between circulating influenza virus strains and bacterial

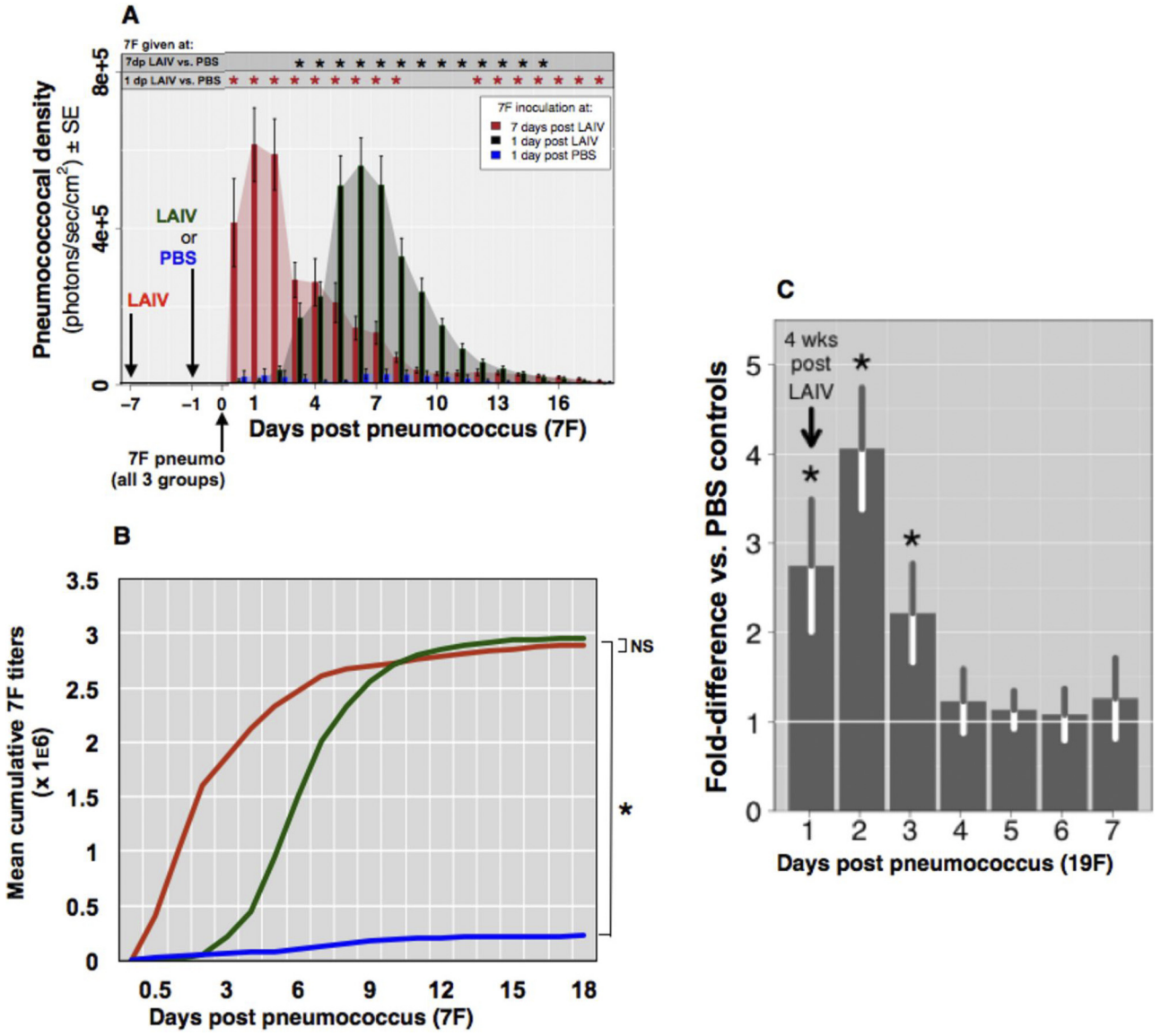

FIG 3 LAIV enhancement of pneumococcal density is time dependent and long lasting. Groups of 12 to 14 mice were vaccinated with LAIV or PBS vehicle at 1 or 7 days prior to colonization with pneumococcal (pneumo) serotype 7F. Bacterial strains constitutively expressed luciferase, and bacterial NP density was measured via IVIS *in vivo* imaging (A and B). Mean cumulative bacterial titers in panel B were calculated by first calculating the cumulative bacterial titers per individual mouse NP at each time point and then calculating the average and SE across the individual cumulative titers per time point, rather than simply averaging the areas under the mean density curves shown in panel A. Asterisks indicate significant differences in bacterial densities between the vaccinated and PBS control groups (dark green indicates LAIV given 7 days prior and red indicates LAIV given 1 day prior to 7F inoculation; $P < 0.05$ by two-tailed Student's *t* test). (C) Groups of mice were vaccinated with LAIV ($n = 20$) or PBS vehicle control ($n = 30$), respectively, at 28 days prior to colonization with 19F pneumococcus. Fold differences per day between mean bacterial densities measured in mice treated 28 days prior with LAIV versus PBS are reported. Error bars indicate standard errors of the mean and asterisks indicate significant differences ($P < 0.05$) from PBS controls (by two-tailed single-sample *t* test).

lower respiratory tract infections (LRIs) (11, 42), we assessed the effects of LAIV on bacterial LRIs and mortality and compared these effects to those seen following WT influenza virus-bacterial coinfection and single infections with bacteria. Mice received LAIV, WT influenza virus, or PBS control and 7 days later (a time known to maximize the lethal effects of influenza virus-bacterial coinfections [43]) were inoculated with a sublethal dose of either of the highly invasive type 2 or 3 pneumococcal serotypes D39 or A66.1, respectively (Fig. 5A to C).

In contrast to the 100% mortality observed when sublethal inoculation with D39 or A66.1 followed pretreatment with wild-type influenza virus, bacterial inoculation following pretreatment with LAIV demonstrated no increases in morbidity (i.e., weight loss; data not shown) or mortality (Fig. 5B and C) relative to bacterial infection alone.

DISCUSSION

The potent and often lethal effects of an antecedent influenza virus infection on secondary bacterial disease have been reported previously (11, 21, 44–46). Viral replication induced epithelial and mucosal degradation, and the ensuing innate immune response yield diminished capacity to avert secondary bacterial infections.

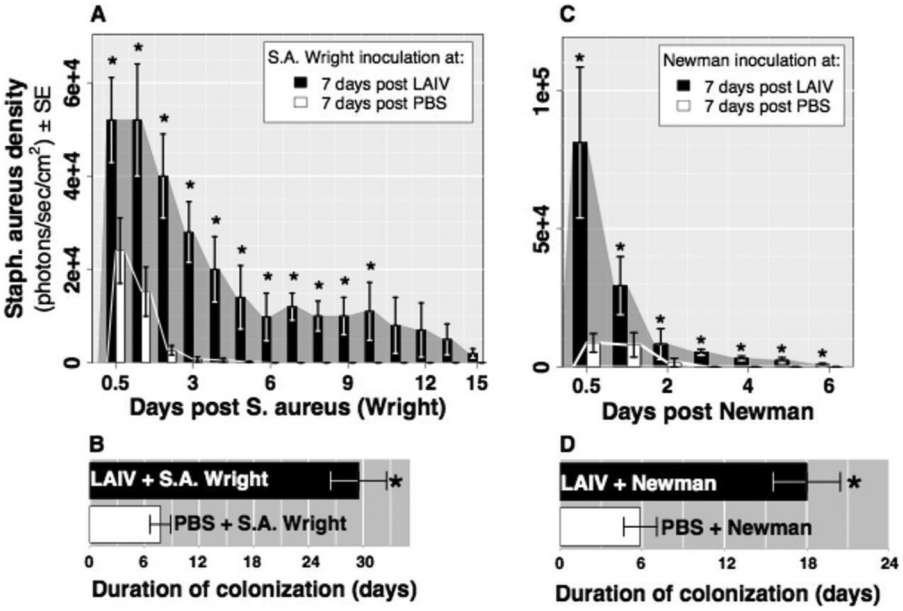

FIG 4 LAIV enhances bacterial load and duration of staphylococcal carriage. Groups of 12 to 14 mice were vaccinated with LAIV or PBS vehicle 7 days prior to colonization with *S. aureus* (S.A.) strain Wright (A and B) or Newman (C and D). *S. aureus* constitutively expressed luciferase, and bacterial density was measured via IVIS *in vivo* imaging. Duration of colonization (B and D) was measured via bacterial plating of nasal washes taken daily after the carriage density decreased below the limit of detection for IVIS imaging. Asterisks indicate significant differences between vaccinated and control groups ($P < 0.05$ by two-sided Student's *t* test), and error bars represent standard errors around the mean.

Recent clinical and experimental data suggest that influenza virus infection may exert its influence beginning in the URT by enhancing susceptibility to bacterial colonization (14, 47, 48) and increasing NP carriage density (36).

Although vaccination with LAIV, in the longer term, thwarts secondary bacterial infections by inhibiting primary infections with influenza virus (29, 49), the immediate effects of LAIV on bacterial replication and disease have never before been described. Indeed, although vaccines are among our greatest achievements in the constant battle against microbial pathogens, the effects of vaccination on distinct pathogen species unrelated to vaccine-targeted pathogens have, until now, remained entirely unexplored. LAIV viruses selectively replicate in the URT, partially denude the epithelium (50), and induce robust innate immune responses that ultimately contribute to long-term protective immunity (28). In so doing, LAIV viruses may, like WT influenza viruses, condition the site of replication for enhanced secondary bacterial colonization.

Here, we demonstrated that vaccination with LAIV, like a WT influenza virus, induces swift increases in bacterial density within the URT, with no discernible differences in effects on bacterial dynamics in the NP between the two virus strains. A lag between viral inoculation and excess bacterial replication of at least 3 to 5 days was consistently measured, no matter the bacterial strain. Of particular interest, the type I interferon, IFN-β, known to play a pivotal role in excess pneumococcal colonization following WT influenza virus infections (36), was maximally upregulated at 3 days post-LAIV vaccination, coincident with commencement of excess bacterial proliferation. After the 3- to 5-day threshold following vaccination was met, the murine NP remained conditioned for excess pneumococcal replication for at least 28 days (our furthest time point out) post-vaccination. However, as the delay between vaccination and bacterial infection was increased, the magnitude of the effects of vaccination on bacterial dynamics became considerably more modest, although statistically significant excess growth was measured even when acquisition followed 28 days post-vaccination.

While the studies described here are limited in scope to murine models, enhanced bacterial load in the URT following LAIV may agree with human data (51), where LAIV has been associated with increases in adverse upper respiratory tract symptoms. Although adverse URT symptoms following administration of FluMist are considered to be of viral etiology, they are most evident in children <5 years of age, where rates of bacterial carriage are greatest (52). Potentially corroborating this are data from a large prospective double-blind trial of FluMist (trial no. MI-CP111 [53]) that assessed reactogenicity and adverse URT events within the first 28 days following vaccination in ~3,000 children between the ages of 6 and 59 months. This trial demonstrated a bimodal increase in URT symptoms following FluMist vaccination, the first between days 2 and 4 post-vaccination and the second between days 5 and 10 post-vaccination (53). While these increased URT events (relative to controls receiving trivalent inactivated influenza vaccine) were considered normal reactions to the live vaccine, the bimodal nature of the increased symptoms suggests that two distinct mechanisms may be in place. In the context of the current findings, the first peak may correspond with viral replication, while the second, more sustained peak may, at least in part, be driven by symptoms due to excess bacterial carriage.

Perhaps the most important finding from our study, with regard to the health of the public and potential concerns regarding vaccination, is that LAIV did not enhance lower respiratory tract infections, morbidity, or mortality following bacterial infections, which are, by most accounts, the most significant issues to be

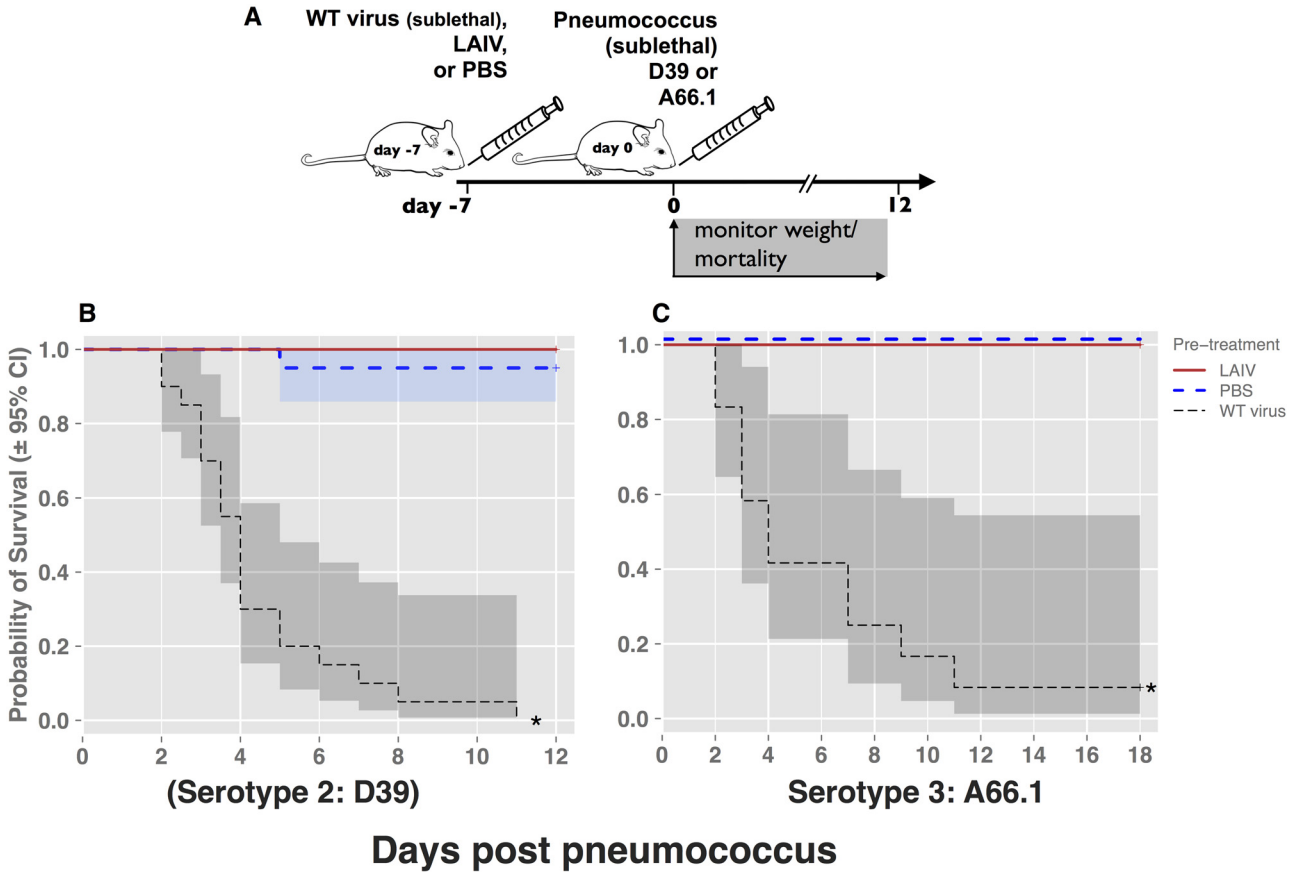

FIG 5 LAIV does not increase severe bacterial disease or mortality. Groups of mice received intranasal LAIV vaccination (solid red curves), sublethal infection with WT influenza virus (broken black curves), or PBS (broken blue curves) 7 days prior to inoculation with a sublethal dose of *Streptococcus pneumoniae* type 2 (1e5 CFU D39; $n = 20$ per group) (B) or type 3 (1e3 CFU A66.1; $n = 12$ to 15 per group) (C), and body weight and mortality were observed at least every 12 h for the first 4 days postpneumococcal inoculation and daily thereafter. Kaplan-Meier survival curves with 95% confidence intervals (CI) were constructed, and asterisks indicate statistically significant differences ($P < 0.05$ by log rank test) between LAIV- or WT virus-infected groups versus PBS controls.

concerned with in terms of respiratory tract bacterial disease. Indeed, this finding is consistent with numerous epidemiological reports all failing to detect any serious adverse sequelae of LAIV vaccination in humans (51, 54). Furthermore, this finding is consistent with significantly diminished LAIV virus replication within the lower respiratory tract, suggesting that viral replication is a requirement for the synergistic response seen between WT influenza viruses and bacterial LRT infections.

While care should be taken to not overgeneralize the data described here to all vaccines, the broad implications suggest that live attenuated viral vaccines may have unintended consequences on important human bacterial pathogens unrelated to the vaccine target species. Furthermore, our findings suggest a role for laboratory models of multispecies interactions with vaccine strains to inform future vaccine monitoring and evaluation programs aimed at identifying thus far entirely unrealized "unconventional" effects, both beneficial and detrimental, of live attenuated viral vaccines and cross-species microbial dynamics.

MATERIALS AND METHODS

Infectious agents and vaccines. Viral infections were carried out with an H3N2 1:1:6 reassortant virus developed as described previously (30), containing the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) from A/Hong Kong/1/68 (HK68) and A/Sydney/5/97 (Syd97) isolates, respectively, and the six internal protein gene segments from A/Puerto Rico/8/34 (or PR8; referred to here as WT influenza virus). LAIV vaccinations consisted of a temperature-sensitive (*ts*) attenuated variant of HK/Syd, HK/Syd$_{att/ts}$ (LAIV) that contains site-specific mutations in the PB1 and PB2 RNA segments of the genome (see Fig. S1 in the supplemental material) as described previously (30). These are the same mutations found in the attenuated A/Ann Arbor/6/60 master donor strain used to produce the influenza A virus strains found in the commercial product FluMist (30). WT and LAIV viruses were propagated in 10-day-old embryonated chicken eggs at 37 and 33°C, respectively) and characterized in Madin-Darby canine kidney cells to determine the 50% infective tissue culture dose (TCID$_{50}$) in wells. The pneumococcal carrier isolates ST425 (serotype 19F) and ST191 (serotype 7F), chosen based on their colonizing potential as previously described (14), were used for colonization experiments. The highly invasive type 2 and type 3 pneumococcal isolates D39 and A66.1, respectively, were used for pneumonia and survival studies. The 19F and 7F strains were engineered to express luciferase, as described previously (14). *Staphylococcus aureus* strains Wright (ATCC 49525) and Newman (ATCC 25905) were engineered to express luciferase by Caliper Life Sciences (Alameda, CA).

Ethics statement. All experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol no. 353) at St. Jude Children's Research Hospital (SJCRH) under relevant institutional

and American Veterinary Medical Association guidelines and were performed in a biosafety level 2 facility that is accredited by the American Association for Laboratory Animal Science (AALAS).

Animal and infection models. Eight-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments, with the exception of mice treated with early vaccination to demonstrate vaccine efficacy and effectiveness. In these cases, 4-week-old BALB/c mice were vaccinated or administered PBS and monitored for 4 weeks before further inoculation. All inoculations and vaccinations were via the intranasal route under general anesthesia with inhaled 2.5% isoflurane (Baxter Healthcare, Deerfield, IL). LAIV vaccination consisted of 2e6 $TCID_{50}$ HK/$Syd_{att/ts}$ LAIV in 40 μl PBS. The lethal and sublethal doses of WT HK/Syd were 5e7 and 1e5 $TCID_{50}$ in 50 μl PBS, respectively. Pneumococcal infections with 19F and 7F were performed as described previously (14), except inoculation was in 40 μl PBS. Infection with *S. aureus* strains Wright and Newman contained 1e7 CFU in 40 μl PBS. Mortality studies were performed as described previously (43) with sublethal doses of the invasive type 2 and type 3 pneumococcal serotype D39 and A66.1 isolates, consisting of 1e5 and 1e3 CFU in 100 μl PBS (to ensure bacterial entry into the lower lungs), respectively. Animals were monitored for body weight and mortality at least once per day for all survival studies. Mice were sacrificed if body weight fell below 70% initial weight.

Bacterial CFU titers for duration studies. 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. Washes were performed daily only after the pneumococcal density fell below the limit of detection for IVIS imaging (~1e4 CFU/ml).

Determination of bacterial and viral titers in lungs and nasopharyngeal homogenates. Viral and bacterial titers were measured in whole lung and nasopharyngeal (NP) homogenates. Whole lungs were harvested and homogenized using a gentleMACS system (Miltenyi Biotech), as per the manufacturer's protocol. NP was isolated via careful dissection dorsally across the frontal bones, laterally via removal of the zygomatic bone, posteriorly by dislocation of the upper jaw from the mandible, and inferiorly just posterior to the soft palate. Isolated NP was homogenized via plunging in 1.5 ml PBS through a 40-μm-mesh strainer. Bacterial titers were measured via plating of serial dilutions, and viral titers were measured by determining the $TCID_{50}$ as previously described (30).

Determination of cytokine levels in the NP and BAL specimens by enzyme-linked immunosorbent assay. Nasopharyngeal isolates and BAL specimens were collected as described above, and cytokines were measured using commercially available kits from R&D systems (macrophage inflammatory protein 1β [MIP-1β], transforming growth factor β [TGF-β], and beta interferon [IFN-β]) or eBiosciences (interleukin-4 [IL-4], IL-6, IL-10, IL-17, IL-23, and gamma interferon [IFN-γ]).

Bioluminescent imaging. Mice were imaged using an IVIS charge-coupled device (CCD) camera (Xenogen) as described previously (14, 29). Nasopharyngeal bacterial density was measured as total photons/s/cm^2 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 two pictures, one for each side of the mouse head. Quantitation was performed using LivingImage software (version 3.0; Caliper Life Sciences) as described previously (14).

Statistical analyses. All statistical analyses were performed within the R statistical computing environment (version 2.14R; R Foundation for Statistical Computing, R Development Core Team, Vienna, Austria). The specific statistical tests used are as indicated in the legend to each figure. The R package Survival was used for all survival analyses, Kaplan-Meier (KM) plots, and KM log rank tests. All other statistical tests were performed using R base functions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01040-13/-/DCSupplemental.

Figure S1, TIFF file, 0.2 MB.

Figure S2, TIFF file, 0.3 MB.

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