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A Novel R848-Conjugated Inactivated Influenza Virus Vaccine Is Efficacious and Safe in a Neonate Nonhuman Primate Model

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Influenza virus infection of neonates poses a major health concern, often resulting in severe disease and hospitalization. At present, vaccines for this at-risk population are lacking. Thus, development of an effective vaccine is an urgent need. In this study, we have used an innovative nonhuman primate neonate challenge model to test the efficacy of a novel TLR 7/8 agonist R848-conjugated influenza virus vaccine. The use of the intact virus represents a step forward in conjugate vaccine design because it provides multiple antigenic targets allowing for elicitation of a broad immune response. Our results show that this vaccine induces high-level virus-specific Ab- and cell-mediated responses in neonates that result in increased virus clearance and reduced lung pathology postchallenge compared with the nonadjuvanted virus vaccine. Surprisingly, the addition of a second TLR agonist (flagellin) did not enhance vaccine protection, suggesting that combinations of TLR that provide increased efficacy must be determined empirically. These data support further exploration of this new conjugate influenza vaccine approach as a platform for use in the at-risk neonate population. ***The Journal of Immunology*****, 2016, 197: 555–564.**

Influenza virus infection is a significant cause of morbidity and mortality in young children, with young infants being at particular risk. The rate of LRTI-associated hospitalizations is more than four times higher in children <1 y of age compared with those between 1 and 4 y (1), and infants <6 mo of age are particularly vulnerable to the development of severe disease (2). Not surprisingly, then, the highest risk for death occurs in the first years of life (2). Infection with this virus can promote a variety of disease states in children <1 y of age including otitis media, pneumonia, myositis, and laryngotracheitis.

The ability to induce protective immunity through vaccination would arguably be the most effective strategy to protect this at-risk population. However, the available data show the rate of seroconversion against H1N1 strains in young infants is only 29–32% after two doses of vaccine (3, 4). Not surprisingly, a correlation was observed between age and seroconversion, with older infants converting at a higher rate than younger infants (3). These data support the critical need for approaches that will improve efficacy in the newborn/infant population.

A case can be made that an optimal vaccine against influenza should induce both cell-mediated and humoral immunity. $CD8^+$ T cells have been documented to play an important role in protection from secondary exposure to infection, for example, after vaccination (5). A clear benefit of $CD8^+$ T cells is their frequent specificity for highly conserved internal viral proteins, which promotes the capacity to provide cross-protection (6). Data suggest generation of this response may be hampered in infants because those dying of influenza virus infection have been reported to have a paucity of $CD8^+$ T cells in their lungs (7).

$CD4^+$ T cells are also a critical component of viral control. The ability of $CD8^+$ T cells to promote clearance of influenza virus in animal models is dependent on help provided by $CD4^+$ T cells (8), and $CD4^+$ T cells are a critical regulator of high-affinity Ab generation postvaccination (9), another important contributor to protection. In this regard, Th1 cells have been reported to promote a more effective Ab response compared with Th2 cells (10–12).

The neonatal immune system presents a number of significant challenges with regard to elicitation of protective Ab- and cell-mediated responses. Ab responses exhibit defects in high-level, high-affinity IgG production through the first year of life (13, 14). Further, there are data in mice (15–18), human cord blood (19), and nonhuman primates (M.A. Alexander-Miller, unpublished data) that show a propensity for differentiation of $CD4^+$ T cells into Th2 cells. Finally, in human neonates, there is evidence supporting a generalized defect in T cell responsiveness (14, 20–25), as well as a heightened regulatory T cell (Treg) response (26, 27).

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Abbreviations used in this article: AGM, African green monkey; BAL, bronchial alveolar lavage; CRP, C-reactive protein; DC, dendritic cell; EID_{50}, egg infectious dose; flg, flagellin; HA, hemagglutinin; i.n., intranasal; NHP, nonhuman primate; pb, postboost; pc, postchallenge; qRT-PCR, quantitative real-time PCR; RT-PCR, real-time PCR; Treg, regulatory T cell.

The impaired immune response in infants makes the development of effective vaccine strategies particularly challenging. Significant effort has been expended toward harnessing the power of TLR agonists for adjuvants with promising results in adults (for review, see Ref. 28). An increasing body of work supports direct conjugation of a TLR agonist to an Ag or synthesis as a fusion protein as mechanisms to improve responses postvaccination (for review, see Ref. 29). TLR agonists that have been used in this way include Pam2Cys, Pam3Cys, MPL, flagellin (flg), imidazoquinolines, and CpG. The proposed advantage of direct linkage lies in targeting of the immunostimulatory component and the Ag to the same cell.

Although this approach results in increased immunogenicity, it is limited in that only one Ag is available for elicitation of immune responses. In this study, we used a novel approach that allows for generation of responses against an array of influenza virus Ags via conjugation of R848 to the intact virion. R848 is an imidazoquinoline compound that has potent stimulatory capabilities for both TLR7 and TLR8. In experimental settings, R848 (or its closely related analog 3M-012) has been shown to increase cell-mediated immune responses when incorporated into HBsAg (30) or HIV gag (31) protein vaccines. R848 has also been reported to induce Ab production (32, 33). The finding that TLR8 agonists induce robust Th1 cytokines in neonatal APC (34) coupled with the reported ability to suppress Tregs (35) makes R848 a highly attractive candidate adjuvant for neonates.

In our studies, an amine derivative of the TLR7/8 agonist R848 was conjugated to influenza virus (IPR8-R848) to test the hypothesis that such a vaccine would result in a robust adaptive immune response in very young infants. In addition, we tested the ability of addition of the TLR5 ligand flg to augment the response given previous studies showing that simultaneous engagement of several TLRs can alter the immune response in both a qualitative and a quantitative fashion (36, 37). The results from our studies show R848-conjugated inactivated influenza virus particles comprise a vaccine that induces robust Ab- and cell-mediated immune responses in neonates. These responses are associated with lessened pulmonary pathology and improved clearance after virus challenge.

Materials and Methods

Animals

African green monkey (AGM) infants (Caribbean-origin *Chlorocebus aethiops sabaeus*) used in this study were housed at the Vervet Research Colony at Wake Forest School of Medicine. Infants were removed from their mothers at 1–3 d of age and moved to the nursery. Infants were initially housed in incubators and subsequently moved to caging when they were capable of thermoregulation. Animal health was assessed by monitoring body weight, temperature, respiration rate, heart rate, food intake, and activity throughout the experiment. Animals were allowed to acclimate to the nursery for 3 d before receiving the vaccine.

Influenza A/PR/8/34 (H1N1)

A/Puerto Rico/8/1934 [H1N1] (PR8) virus stocks were grown and titered (egg infectious dose [EID_{50}]) in 10-d-old fertilized chicken eggs (obtained from a local farm) essentially as described previously (38). Stocks were diluted in PBS, flash frozen, and stored at −80°C.

Vaccination

At 4–6 d of age, infants were vaccinated with 45 μg inactivated R848-conjugated virus (IPR8-R848) in the presence or absence of 10 μg flg or with inactivated IPR8 (IPR8) mixed with the same amount of the inactive 229 mutant flg (m229) (39). This dose of flg has previously been shown to be effective in young AGM (40). Control animals received PBS. Inactivation of PR8 was achieved by treating with 0.74% formaldehyde overnight at 37°C. Virus was dialyzed against PBS and tested to assure the absence of infectivity. For the IPR8-R848 conjugate vaccine, an amine derivative of R848 (heretofore referred to as R848) was linked to SM (PEG)4 (Thermo Scientific) by incubation in DMSO for 24 h at 37°C (Supplemental Fig. 1). R848-SM(PEG)4 was then incubated with influenza virus that had been reduced to generate free thiol groups (IPR8-R848). Unconjugated R848 was removed by extensive dialysis. This construct was then inactivated by treatment with 0.74% formaldehyde overnight at 37°C, followed by dialysis. Successful conjugation was assessed by differential stimulation of RAW264.7 cells (Wake Forest Comprehensive Cancer Center Cell and Viral Vector Core Laboratory) after incubation with similar amounts of R848-conjugated versus nonconjugated vaccine. flg from *Salmonella enteritidis* was prepared as previously described (39). In brief, *E. coli* BL21 (DE3) containing a pet29a::*fliC* encoding wild type flg or the truncated pet29a::229 encoding only the biologically inactive hypervariable region of flg (39, 41) were grown and lysates prepared in 8 M urea. Proteins were purified on Ni-NTA agarose (Qiagen) according to the manufacturer's protocol. Endotoxin and nucleic acids were removed using an Acrodisc Mustang Q capsule (Pall Corporation). Purified proteins were extensively dialyzed against PBS. All injections were delivered i.m. in the deltoid muscle (500 μl vol). Animals were boosted 21 d later. Seven infants received IPR8-R848, seven received IPR8-R848+flg, four received IPR8+m229, and three received PBS.

Virus challenge and sampling

On days 23–26 postboost (pb), animals were sedated with 2–5% inhalant isoflurane. Animals received 1×10^{10} EID_{50} PR8 divided equally between the intranasal (i.n.) and intratracheal routes, 0.25 ml intratracheally and 0.25 ml i.n. (0.125 ml/nostril). For subsequent sampling, animals were sedated with isoflurane. Blood was collected in sodium heparin tubes by venipuncture on days 8 and 14 postchallenge (pc), plasma obtained by centrifugation, and PBMC subsequently isolated using Isolymph. Tracheal washes were performed on sedated animals on days 2, 5, 8, and 14 postinfection by inserting an endotracheal tube into the trachea, instilling sterile 1.0 ml PBS and aspirating back. Due to the small volume of PBS used in the infants, 0.5 ml PBS was used to wash out the endotracheal tube. Samples were centrifuged to remove cellular material and BSA was added to a final concentration of 0.5%. Bronchial alveolar lavage (BAL) was performed at necropsy (day 14) using 5 ml PBS. Samples were centrifuged to remove cellular material, and BSA was added to a final concentration of 0.5%.

Assessment of lung pathology

Sections of lung were preserved in 10% neutral buffered formalin for at least 24 h, trimmed, embedded in paraffin, and routinely processed for histology. Sections were cut at 6 μm and stained with H&E. The slides were examined by light microscopy by an American College of Veterinary Pathologists board-certified veterinary pathologist in a blinded fashion and evaluated for degree of inflammation and injury. Pathology assessment was based on interstitial and alveolar inflammatory cell infiltration and edema, pneumocyte hyperplasia, and bronchial degeneration and necrosis.

Quantitation of viral load

Viral RNA was extracted from the tracheal samples using QIAamp Viral RNA Mini Kit (Qiagen). cDNA was synthesized from mRNA by reverse transcription using Superscript III RT kit (Invitrogen) and random primers (Invitrogen). For viral quantification, RNA primer-probe sets specific for H1N1 were used (BEI Resources). Quantitative real-time PCR (qRT-PCR) was performed using the Applied Biosystems 7500 real-time PCR system. EID_{50} values were calculated based on a standard curve generated using a stock of known EID_{50}. The total EID_{50} for the sample was calculated based on the amount present in the sample volume used for the RT-PCR (140 μl) and adjusting to the total volume used for the wash.

C-reactive protein measurement

C-reactive protein (CRP) levels in the plasma were assessed at 24 h postvaccination using a C-Reactive Protein ELISA kit from ALPCO Diagnostics as per the manufacturer's instructions. The plate was read at 450 nm on a BioTek Elx800 Absorbance Microplate Reader. Amounts were calculated based on the standard curve generated using the control provided in the kit.

ELISA for the detection of influenza virus-specific Ab

Nunc MaxiSorp ELISA plates were coated with 1 μg/well PR8 or 0.2 μg/well recombinant hemagglutinin (HA; BEI Resources) in sodium carbonate/bicarbonate coating buffer (pH 9.5). Plates were blocked with 1× Blocking Buffer (10× Blocking Buffer; Sigma) plus 2% goat serum (Lampire Biologicals) and washed. The wash buffer used throughout the assay was PBS with 0.1% Tween 20. Plasma or respiratory samples were serially diluted in 1× Blocking Buffer. Wells without virus served as a negative control.

HRP-conjugated Ab specific for monkey IgG (Fitzgerald) or IgM (LifeSpan Bioscience) was used to detect bound Ab. Plates were developed with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma) and read at 450 nm on a BioTek Elx800 Absorbance Microplate Reader. Absorbance for each dilution was calculated by subtracting the OD value obtained for the corresponding nonvirus-coated wells. Threshold titer was defined as the value that reached three times the assay background, that is, wells that received only sample diluent.

Neutralization assay

Heat-inactivated (56°C for 1 h) samples were serially diluted in a sterile 96-well flat-bottom plate. A total of 7.5×10^6 EID_{50} PR8-GFP (kindly provided by Dr. Adolfo García-Sastre [42]) was added to each well and incubated for 2 h at 37°C and 5% CO_2 to allow for Ab binding. A total of 2×10^5 U937 cells (Wake Forest Comprehensive Cancer Center Cell and Viral Vector Core Laboratory) was then added to each well and incubated overnight at 37°C. The next morning, samples were acquired on a BD FACSCalibur and analyzed with CellQuest Pro software (Becton Dickinson) to determine the percentage of U937 cells that were positive for GFP. Controls for each experiment consisted of U937 cells alone and U937 cells infected with the PR8-GFP virus in the absence of plasma. Maximal %GFP was calculated for each experiment, and nonlinear regression (GraphPad Prism) was used to determine the dilution at which the 50% maximum PR8-GFP–infected U937 cells was achieved.

T cell ELISPOT

Dendritic cells (DCs) were generated from bone marrow isolated from the animals in the study by culture in the presence of GM-CSF (40 ng/ml) and IL-4 (40 ng/ml) for 6 d. Bone marrow was collected under an approved protocol. Differentiation was assessed by flow cytometric staining for CD11c. DCs were infected with GFP-PR8 virus (42), and successful infection was determined by flow cytometric analysis. Infected or mock-infected DCs were cocultured in triplicate with autologous cells for 48 h in ELISPOT plates coated with anti–IFN-γ (GZ-4) or anti–IL-4 (IL-4-I) capture Ab (MABTECH). After incubation, spots were detected using biotin-conjugated anti–IFN-γ (7-B6) or anti–IL-4 (IL-4-II) detection Ab (MABTECH), streptavidin-HRP conjugated Ab, and TrueBlue substrate solution. Spots were analyzed by ImmunoSpot Analyzer (Cellular Technology) and ImmunoSpot (version 3.2) software. The number of cytokine secreting cells per tissue was calculated based on the total number of cells recovered.

Statistical analysis

For continuous outcomes, groups were compared using two-sample *t* tests (if the treatment group had two levels) or ANOVA models (if there were three or more levels for the treatment group), with specific contrasts defined to compare pairs of groups when appropriate. Prior to fitting the statistical models, outcome data were examined to determine if they followed normal distributions. If an outcome was found not to be normally distributed, the outcome was logarithmically transformed. These log-transformed outcomes were then examined for normality, and if the transformation led to the data being normally distributed, analyses were performed using the transformed data. For analyses that included repeated measures (i.e., for IgG), a two-way repeated-measures mixed model (two-way repeated-measures ANOVA) was fit with the primate considered as a random effect in the model and treatment group and day considered as fixed effects. The treatment group by day interaction was examined first in these models and if found to be nonsignificant, then that term would be removed. Comparisons between pairs of groups or on particular days were performed within these mixed models if the overall group or day effects were found to be significant. Data were analyzed using Prism 5 software (GraphPad) or SAS Version 9.3.

Animal approval

All animal protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine. The Wake Forest School of Medicine animal care and use protocol adhered to the U.S. Animal Welfare Act and Regulations.

Results

Infant vaccination

To assess candidate vaccines for the ability to elicit robust immune responses in young infants, we developed an AGM model of neonate vaccination (43). This serves as a tractable model for the human infant because it provides a period of infancy of adequate length to effectively evaluate prime boost strategies. In addition, this model is advantageous given the similarity of nonhuman primates (NHP) and humans in TLR distribution and function (44). Our experimental vaccine is composed of formalin inactivated influenza PR8 virus (IPR8) adjuvanted through direct conjugation to a synthetic amine derivative of R848, a potent TLR7/8 ligand (Supplemental Fig. 1). In some cases we also used a combination adjuvant approach by including the soluble TLR5 agonist flg, which was shown in our previous studies to possess some potential to enhance both T and B cell recall responses; that is, the effect of flg was restricted to boosting (43). Control animals received inactivated PR8 together with an inactive flg construct (m229, nonadjuvanted control) or PBS (nonvaccinated control).

The overall experimental design is shown in Fig. 1A. Infant AGM received the initial vaccination at 4–6 d of age. Animals were boosted 21 d later and challenged with live influenza virus 23–26 d pb. Infants were monitored for virus load and recall responses pc and necropsied at 14 d pc. To assess safety, we monitored infants for changes in temperature, respiration, heart rate, and overall health every 4 h after receiving the vaccine for 24 h. No consistent change in any of these health indicators was observed (data not shown). Further, there were no local site reactions after vaccination.

As a measure of systemic immune activation/inflammation, CRP levels were assessed at 24 h postvaccination. We fit a one-way ANOVA model to compare the four groups, followed by pairwise comparisons of groups using *t* tests based on contrasts within the ANOVA model. We observed a highly significant ($p < 0.001$) increase in CRP levels at 24 h postvaccination in animals receiving the vaccine with the combination of R848 and flg adjuvants (Fig. 1B). This level was not higher than that observed in our previous study of vaccination with flg as the sole adjuvant (43). Thus, the increase in CRP appears to be mediated predominantly by flg. In agreement with this, we observed only a modest increase in CRP level in IPR8-R848–vaccinated infants compared with those vaccinated with IPR8+m229. The increases in CRP (2.9- to 10.8-fold) are in line with those reported in other vaccine studies (45, 46). There were no significant increases in IL-6 or TNF-α in the vaccinated infants (data not shown), suggesting the absence of a systemic proinflammatory cytokine response.

R848 is a robust adjuvant for generation of an influenza virus–specific Ab response

Circulating levels of influenza-specific IgG and IgM were measured at 10 and 21 d after primary and boost vaccinations. The two-way repeated-measures ANOVA model showed a significant group by day interaction, suggesting that the rate of change in IgG levels was different depending on the group. Based on this significant interaction, we compared the groups at each specific day. The level of anti-influenza virus IgG in vaccinated versus PBS-treated infants was significantly increased in all cases at all times assessed ($p < 0.001$). This is expected and as such significance is not indicated on Fig. 2 for increased ease of viewing. Inclusion of conjugated R848 resulted in a significant increase in virus-specific IgG at day 21 postprimary vaccination, as well as at days 10 and 21 pb compared with animals that received m229 (Fig. 2A). The addition of flg to IPR8-R848 did not result in a significant increase compared with IPR8-R848 alone (Fig. 2A).

Influenza-specific IgM levels were also assessed. We found no evidence for a day by group interaction ($p = 0.92$). Therefore, overall group comparisons could be made across the four time points by adjusting for day rather than by comparing groups separately on each day. The presence of R848 significantly increased the influenza-specific IgM response compared with m229 control animals (Fig. 2B). As with

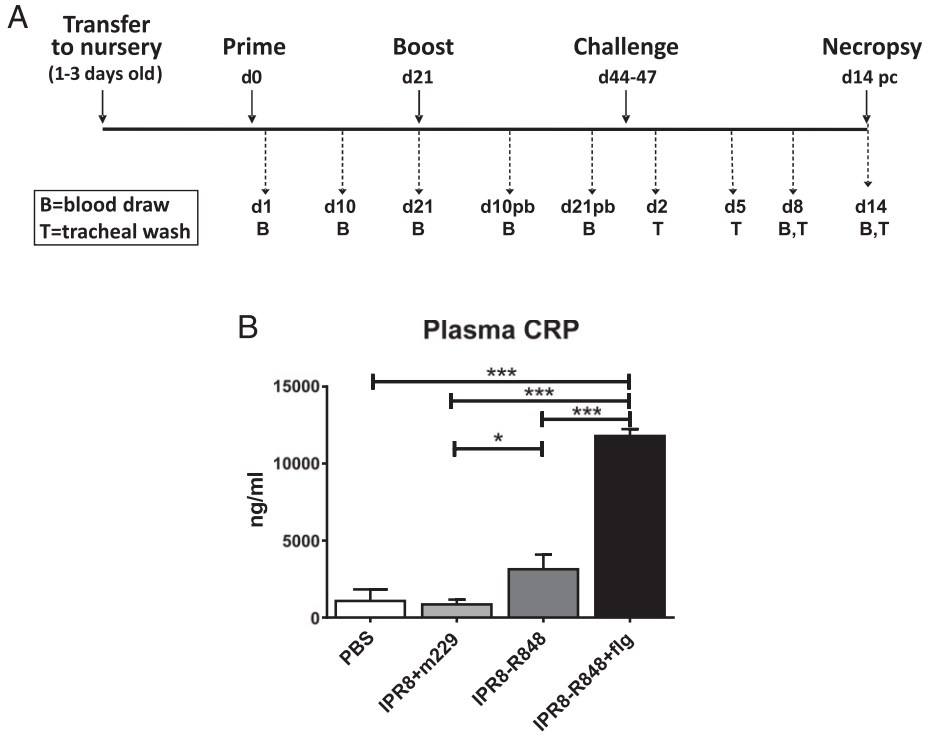

FIGURE 1. Experimental study design and CRP levels after vaccination. (**A**) Neonate AGMs (4–6 d old) were vaccinated (d0) and boosted 21 d later with inactivated PR8 influenza virus (IPR8) together with the inactive form of flg (m229) ($n = 4$) or R848-conjugated IPR8 in the presence or absence of flg ($n = 7$). Nonvaccinated control animals received PBS ($n = 3$). Blood (B) was drawn on days 1, 10, and 21 after initial vaccination and days 10 and 21 after boost. Twenty-three to 26 d after boost, animals were challenged with live PR8 virus (10^{10} EID_{50}). On day 14 pc animals were necropsied. Tracheal wash (T) samples were acquired on days 2, 5, 8, and 14 pc and blood (B) on days 8 and 14 pc. (**B**) Twenty-four hours after initial vaccination, plasma CRP levels were measured. Significance was determined by one-way ANOVA. $*p < 0.05$, $***p < 0.001$.

IgG, there was no significant difference between IPR8-R848 and IPR8-R848+flg. Further, as with IgG, all vaccinated animals had a highly significant increase compared with animals that received PBS ($p < 0.001$). No virus-specific IgA was detected as would be expected after i.m. delivery (data not shown). Together, these results show the presence of R848 conjugated to the virus particle had a robust adjuvant effect for elicitation of Ab in infants postvaccination. The addition of flg to the IPR8-R848 vaccine did not further increase systemic levels of vaccine-induced, virus-specific Ab.

Combination of R848 and flg results in increased neutralizing Ab compared with R848 alone

The presence of neutralizing Ab was measured by the ability to inhibit infection of tissue culture cells by a GFP-expressing influenza PR8 virus. The neutralization titer was defined as the dilution of plasma that inhibited infectivity by 50% compared with infection in the absence of plasma. This approach was chosen because it is a direct measure of the ability of the Ab to prevent infection. The assay was validated against the standard HA inhibition assay, finding that it provided similar relative results (43). The two-way repeated-measures ANOVA model found a significant day by group interaction, and as such pairwise comparisons among groups were performed separately on each day. Statistically significant increases in neutralizing titer were observed at both days pb in infants that received the IPR8-R848+flg versus m229 adjuvanted vaccine (Fig. 2C, circles versus diamonds). Comparison of IPR8-R848+flg– with IPR8-R848–vaccinated infants revealed a significant increase at day 10 pb, but this was lost at day 21 pb. Although it did not reach statistical significance, an increase in the average neutralization titer was observed for animals vaccinated with IPR8-R848 versus IPR8+m229. The failure to obtain significance may be a result of heterogeneity among infants in these two groups. These data suggest R848 induces increases in neutralizing titer and show the combination of R848 and flg can enhance neutralizing Ab at some time points compared to R848 alone.

The higher Ab levels generated in response to R848-conjugated vaccines result in increased levels of Abs that can recognize homologous, but not heterologous, subtype HA molecules

To determine whether the presence of the adjuvants could impact the ability of Ab to recognize additional strains of influenza virus, we assessed recognition of HA molecules derived from the vaccine PR8 strain, A/New Caledonia/20/1999 (H1), A/California/07/2009 (H1), and A/Wisconsin/67/2005 (H3). Plasma from animals that were 21 d pb was used because this was the time at which maximal Ab levels were detected. As expected from the earlier analyses, all vaccines induced Ab that could recognize PR8 HA (Fig. 2D). None of the vaccines elicited Abs capable of recognizing the heterologous H3 molecule from the Wisconsin strain (Fig. 2D). Analysis of the recognition of additional H1 molecules showed vaccination with IPR8 in the absence of adjuvant (IPR8+m229) resulted in detectable Abs to the New Caledonia, but not the California H1 molecule (Fig. 2D). The presence of R848 or R848+flg could promote recognition of the HA molecule from New Caledonia in addition to California. Thus, the presence of R848 or R848+flg was capable of generating an Ab response with expanded capability to recognize HA molecules from other strains (Fig. 2D). These data support inclusion of R848 as a mechanism to promote the presence of Abs with broader cross-strain recognition. This could occur through higher total virus-specific Ab and/or increased capacity for cross-recognition.

R848 promotes a greatly augmented systemic Ab response after virus challenge

An important goal of vaccination is to generate adaptive immune cells that can respond rapidly and effectively after pathogen challenge. To assess the recall potential of the immune response generated in the vaccinated infants, we delivered influenza virus by the combined intratracheal and i.n. routes at 23–26 d pb. On days 8 and 14 pc, the circulating level of virus-specific Ab was measured. At day 8 pc, animals that had received R848-conjugated vaccine exhibited greatly increased levels of virus-specific IgG Ab compared with animals that received the negative control (m229)

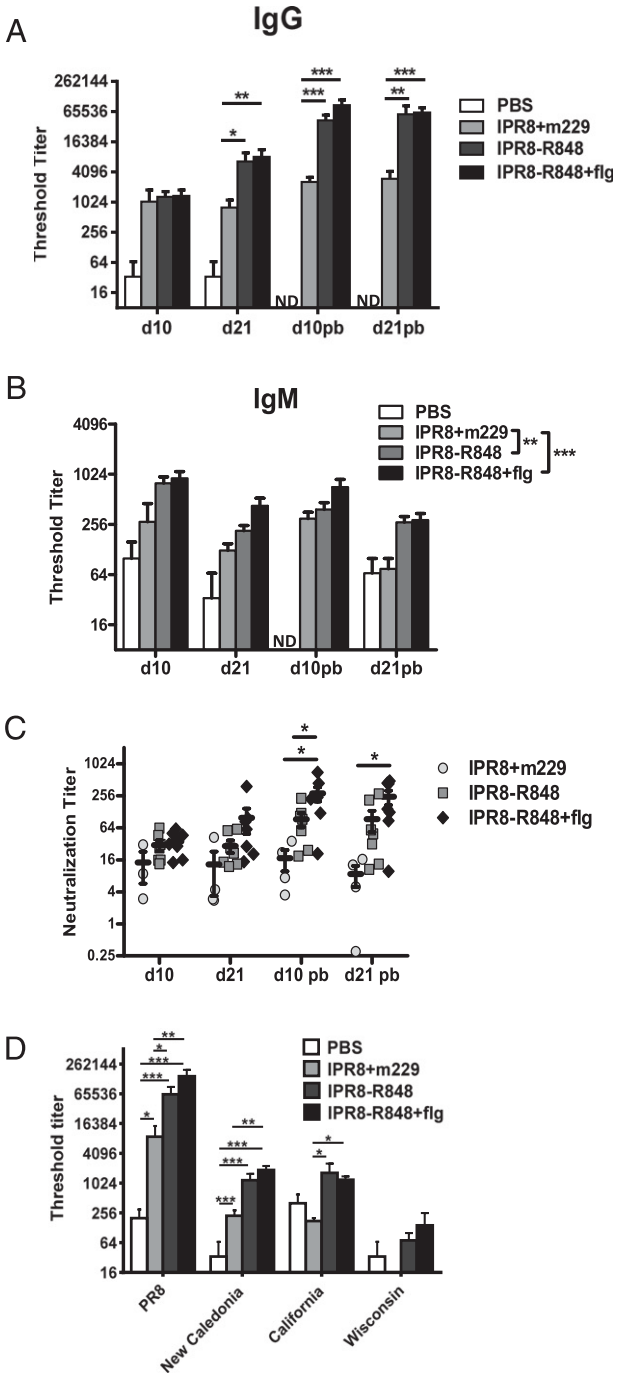

FIGURE 2. Conjugation of R848 to IPR8 results in an increase in total and neutralizing influenza virus–specific Ab as well as Ab capable of recognizing alternative H1 molecules. Levels of PR8-specific IgG (**A**) and IgM (**B**) and neutralizing Ab (**C**) in plasma were measured 10 and 21 d after prime and boost. Averaged data are shown. Significantly increased influenza-specific IgG and IgM responses were observed when R848 was conjugated to inactivated virus. The addition of flg did not significantly improve systemic responses. (**D**) Plasma obtained from infants at day 21 pb was assessed by ELISA for the presence of IgG Abs capable of recognizing the HA molecule from A/New Caledonia/20/1999 (H1N1), A/California/07/2009 (H1N1) pdm09, and A/Wisconsin/67/2005 (H3N2). Significance was determined using a repeated-measures mixed model fit. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ND, not detected.

adjuvanted vaccine (Fig. 3A). Infants vaccinated with IPR8-R848 had, on average, a 10.2-fold increase and those vaccinated with IPR8-R848+flg a 20.6-fold increase compared with infants that received IPR8+m229. Although Ab was increased, the R848+flg group was not significantly different from R848 alone. Virus-specific IgM levels were also increased at this time point (Fig. 3B). At day 14 pc, virus-specific IgG Ab in infants vaccinated with adjuvanted vaccines remained significantly higher than those receiving IPR8+m229 (Fig. 3A). Interestingly, at day 14 pc, infants vaccinated with IPR8+m229 and nonvaccinated infants exhibited similar levels of influenza-specific IgG (Fig. 3A), suggesting the nonadjuvanted vaccine did not induce a recall response pc that was superior to that of a naive infant.

Analysis of the neutralizing potential of virus-specific Ab in plasma revealed a significantly higher titer at day 8 pc in infants that were vaccinated with the two R848-containing vaccines compared with animals vaccinated with IPR8+m229 (Fig. 3C). Those receiving the vaccine that included both R848 and flg had a higher average neutralizing titer, although the difference was not statistically significant compared with R848 alone ($p = 0.08$) (Fig. 3C). Together these data show the presence of R848 during vaccination of young infants resulted in an increase in total virus-specific Ab as well as neutralizing Ab at early times pc, a time point that is likely critical in determining the outcome of infection, for example, disease severity.

Combined presence of R848 and flg results in the highest level of virus-specific respiratory IgG pc

We also assessed the presence of Ab in the trachea (days 8 and 14) and BAL (day 14) pc. Two-way repeated-measures ANOVA analysis of the dataset revealed evidence of nonsignificant day by group interaction ($p = 0.23$). Therefore, for the analysis of tracheal Ab, we compared groups across all four time points by adjusting for day rather than comparing groups on each day. Compared with the IPR8+m229 vaccine group, only the IPR8-R848+flg group resulted in a significantly increased virus-specific IgG response in the trachea (Fig. 4), which was unexpected given the significant increase in systemic virus-specific IgG in the IPR8-R848–vaccinated infants. No significant differences across the groups were detected in the BAL at day 14. Together these data suggest that the combination of flg and conjugated R848 may promote greater virus-specific IgG Ab in the respiratory tract.

Vaccination with R848-conjugated IPR8 results in an increase in IFN-γ–producing T cells pc

We next evaluated the T cell response present in vaccinated animals pc. IFN-γ– and IL-4–producing cells were assessed at day 8 pc in the blood and at day 14 pc in the lung, spleen, and lung-draining tracheobronchial lymph node (Fig. 5). Infants that received either of the R848-conjugated vaccines showed significantly enhanced numbers of IFN-γ–producing cells in the blood at day 8 pc and in all tissues evaluated at day 14 pc. IL-4–producing cells were significantly increased only in day 8 PBMCs of infants vaccinated with the dual-adjuvant vaccine; however, it is noteworthy that the R848-mediated increase in IL-4–producing cells was modest compared with that observed for the IFN-γ–producing population (Fig. 5). Thus, conjugation of R848 to inactivated influenza virus results in a population of cells that exhibits a selective and robust induction of IFN-γ–producing, influenza-specific T cells during the recall response elicited after virus challenge. This enhancement was not further increased by the addition of flg.

Presence of R848 results in improved clearance and reduced pathology pc

A protective vaccine should result in decreased viral burden and reduction in disease pc. To assess the protective capacity of the candidate vaccines, viral load in the trachea was measured over

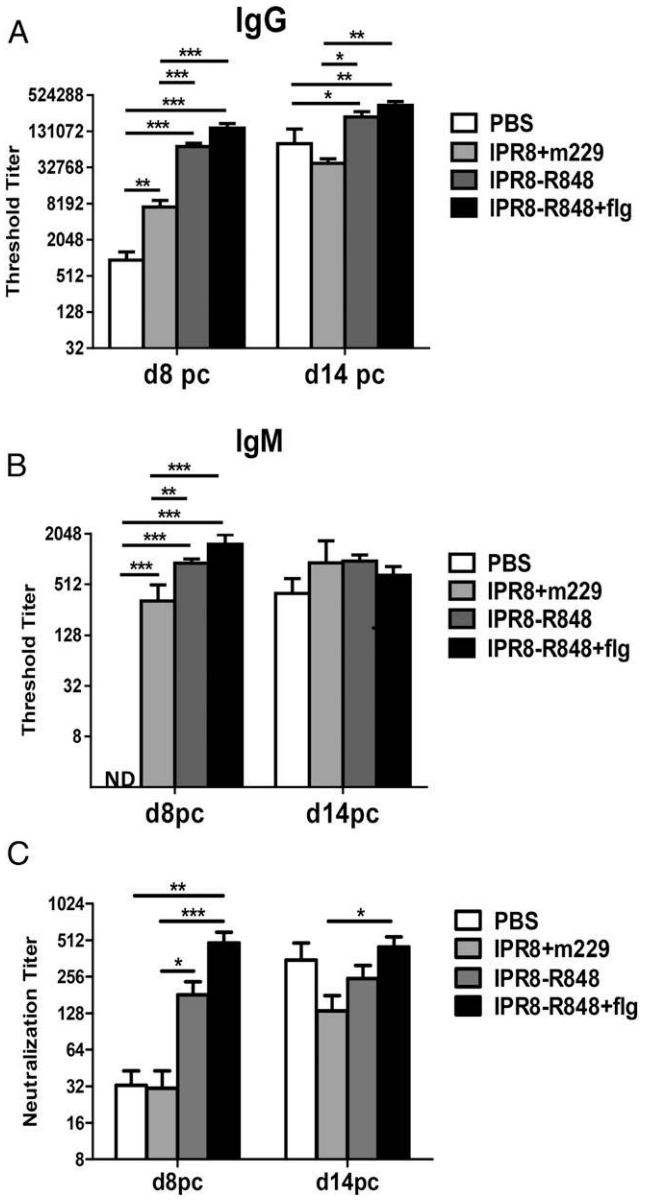

FIGURE 3. IPR8 conjugation with R848 results in an increase in virus-specific total and neutralizing Ab at early times pc. The amount of influenza-specific IgG (**A**) and IgM (**B**) Ab as well as neutralizing titer (**C**) was measured in plasma at days 8 and 14 pc. Significance was determined using a repeated-measures mixed model fit. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ND, not detected.

time by qRT-PCR. Viral load is presented as EID_{50} equivalents based on a standard curve generated using a stock of known EID_{50}, with the acknowledgment that there may be differences in the ratio of infectious virions/RNA in virus obtained from the infants and that grown in vitro. Because the two-way repeated-measures ANOVA analysis suggested no evidence of a day by group interaction ($p = 0.25$), we compared groups across all four time points by adjusting for day rather than comparing groups on each day. Animals vaccinated with the either IPR8-R848 or IPR8-R848+flg exhibited significantly decreased viral load in the trachea (Fig. 6A) and cleared virus earlier compared with the other constructs (Fig. 6B). The addition of flg to the IPR8-R848 vaccine did not significantly enhance clearance. Viral RNA in the lung at day 14 pc was below detection in all groups (data not shown).

We also assessed pathology in the lungs of vaccinated infants at 14 d pc. Lung sections were scored in a blinded fashion by a board-certified veterinary pathologist into three categories: marked, moderate, or mild. Three of the four infants vaccinated with IPR8+m229 had moderate changes pc compared with one of seven IPR8-R848–vaccinated infants (Fig. 6C, 6D). The remainder of infants that received IPR8-R848 showed mild pathology. Unexpectedly, three of seven IPR8-R848+flg–vaccinated infants had moderate changes. Thus, although the Ab response in the respiratory tract of infants that received the combination adjuvant vaccine was increased, the dual adjuvanted vaccine was not associated with less pulmonary pathology or increased viral clearance compared with R848 alone.

Discussion

Impaired immune responsiveness significantly hampers effective vaccination of neonates. Generation of vaccine approaches that can overcome these barriers is critical given the severe disease that often accompanies respiratory virus infection in this population together with the limited number of available therapeutics. To test immunogenicity and efficacy of candidate vaccines against influenza in neonates, we have established an NHP model that is arguably the most relevant system to probe these questions. With this model, we assessed a novel vaccine construct, generated using a methodology developed in our laboratory, which allows conjugation of the TLR7/8 agonist R848 directly to the influenza virus particle. The selection of R848 was made based on the potential to stimulate DCs, B cells, and T cells (34, 47–50), as well as to suppress T regulatory cells (through TLR8) (35). The last is of particular interest given reports showing increased function and/or differentiation of T regulatory cells in neonates (26, 27, 51). Further, although TLR responsiveness is impaired in neonates, in myeloid neonate-derived DCs there is evidence that TLR8 responsiveness is less diminished than that of TLR7 (34). Thus, the dual TLR7/8 targeting by R848 makes it particularly attractive for neonate vaccines. Finally, although previous studies have reported the increased potency of vaccines when TLR agonists are covalently coupled to the Ag (for review, see Fujita et al. [29]), these constructs have the limitation that they use a single antigenic

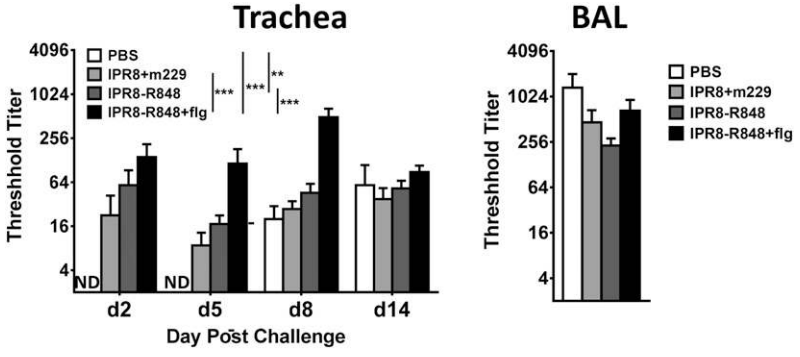

FIGURE 4. The combination of flg and conjugated R848 results in the highest level of virus-specific IgG Ab in the respiratory tract at early times pc. Influenza virus–specific IgG in the trachea was measured at days 2, 5, 8, and 14 pc and in the lung (BAL) at day 14 pc. Significance was determined using a repeated-measures mixed model fit. $**p < 0.01$, $***p < 0.001$. ND, not detected.

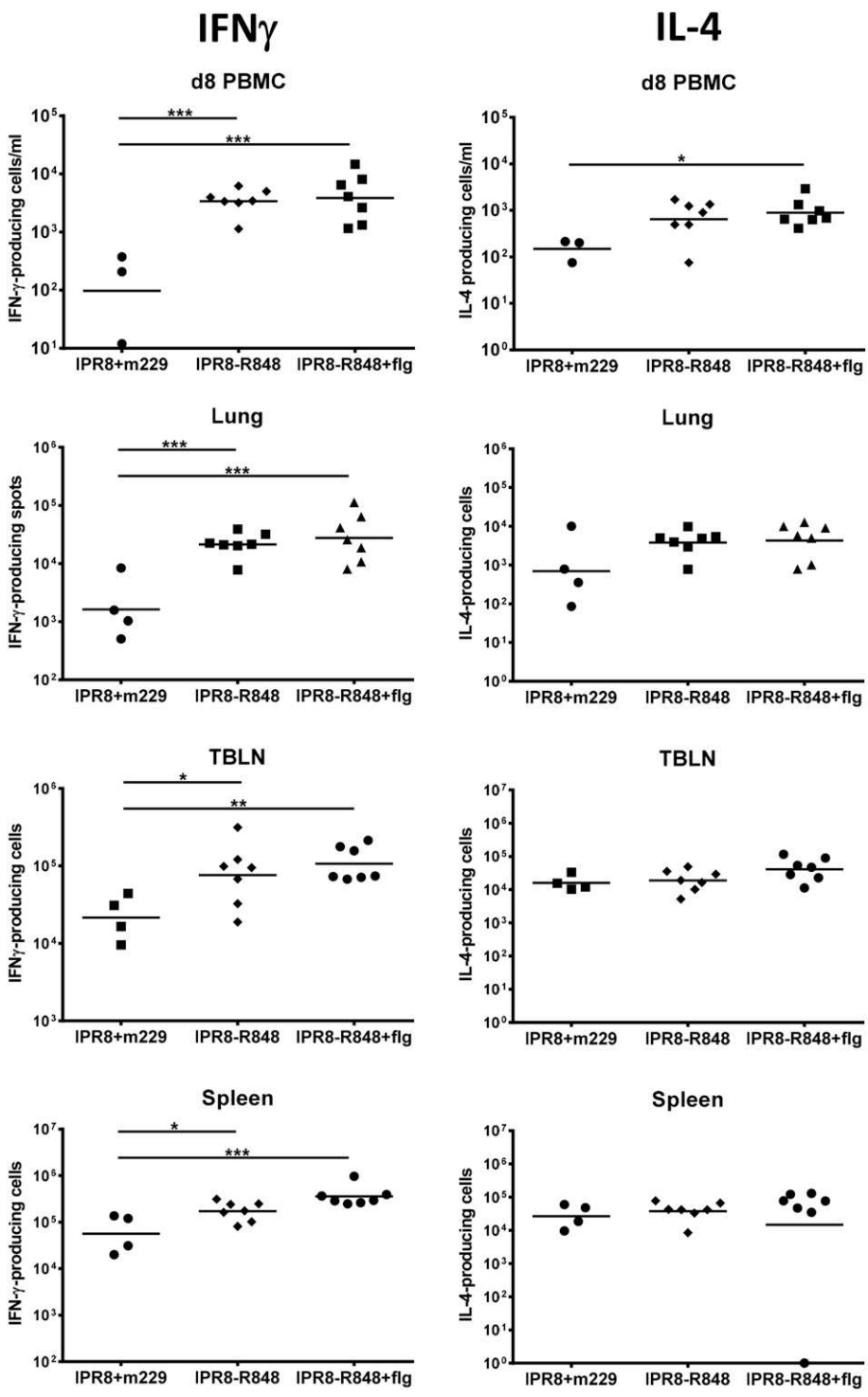

FIGURE 5. Infants vaccinated with IPR8-R848 have more IFN-γ^+ influenza-specific T cells pc. IFN-γ– and IL-4–producing influenza-specific T cells were quantified by ELISPOT in the blood at day 8 pc and in the lung, tracheobronchial lymph node, and spleen at day 14 pc after stimulation in the presence of autologous DCs infected with influenza virus. Individual animals and the geometric mean are shown. Notably, one IPR8+m229 PBMC blood draw at day 8 did not yield an adequate number of cells for analysis. Significance was assessed using one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

target. Thus, the immune response is relatively narrow. In contrast, the vaccine used in our study contains all viral proteins allowing for multiple antigenic targets.

Our results demonstrate conjugation of R848 to the influenza virion results in a highly augmented virus-specific T cell response as well as IgG Ab (both total and neutralizing) compared with nonadjuvanted inactivated virus. Importantly, augmentation of the T cell response is primarily within the IFN-γ–producing population. At present, a limitation of the experimental design used in this study is the inability to determine whether the IFN-γ production is from Th1 versus $CD8^+$ T cells, an area of future interest for assessment. In addition, the extent to which any influenza-specific $CD8^+$ T cells that may be present possess cytolytic activity is not known. Nonetheless, our data show that this vaccine approach promotes generation of the most beneficial immune responses (IFN-γ–producing T cells and Ab) with regard to protection from influenza virus infection.

The increases observed with R848 are significantly greater than those observed in our previous study in which the utility of flg alone was assessed (43). Thus, the novel approach of conjugation of R848 to the virion studied in this article results in a superior vaccine. The ability to markedly enhance IgG responses in these very young infants is extremely promising because Ab production is known to be generally weak for the first year of life (22).

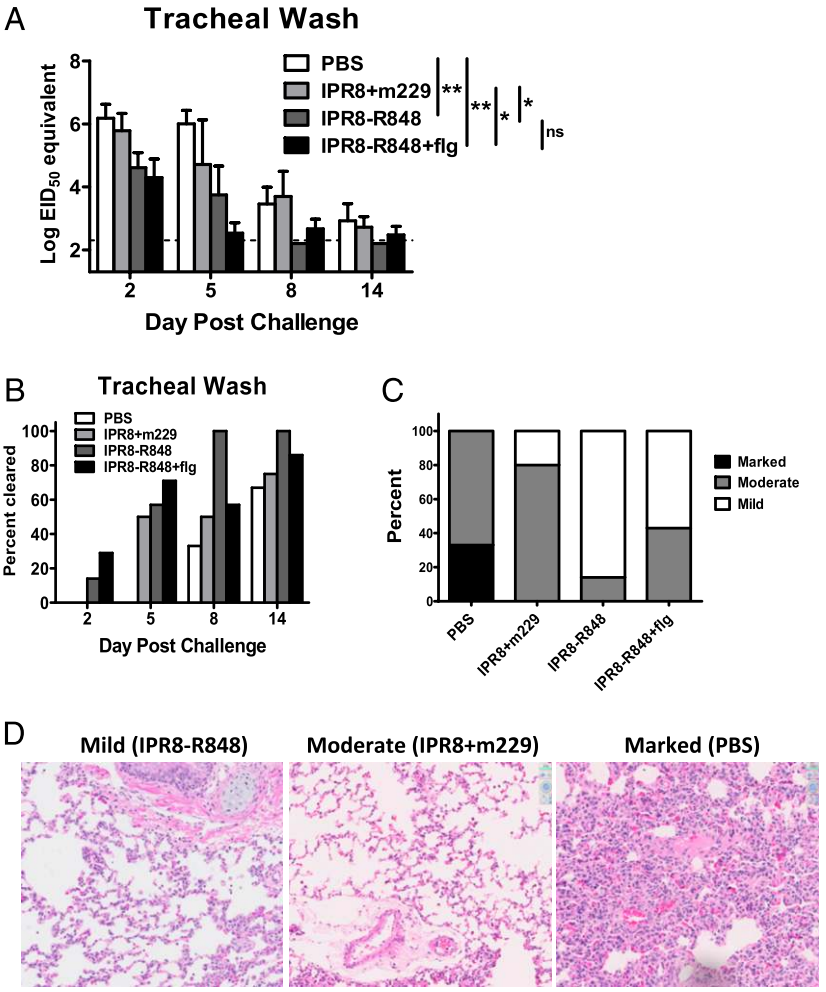

FIGURE 6. Vaccination with R848-conjugated IPR8 results in recall responses that are more protective. (**A**) Virus load in the trachea after virus challenge of vaccinated animals was assessed by qRT-PCR. Virus in the trachea of IPR8-R848–vaccinated infants was reduced pc compared with m229-adjuvanted and PBS animals. Data shown are EID_{50} equivalents calculated based on known concentrations of influenza virus. The dashed line represents the limit of detection. Significance was determined using a repeated-measures mixed model fit. $*p < 0.05$, $**p < 0.01$. (**B**) Data represent the percentage of animals in which virus was below the limit of detection in the trachea at each indicated time pc. IPR8-R848–vaccinated animals show earlier clearance compared with other vaccine groups. (**C**) The severity of lung pathology was determined in a blinded fashion by a board-certified veterinary pathologist. Pathology was assigned as mild, moderate, or marked for each animal. Infants with mild pathology exhibited alveoli that were open and regularly contained few inflammatory cells that were mostly macrophages with few neutrophils. Alveolar septa were mildly thickened in some areas. Connective tissue fibers around vessels were separated by clear space, consistent with edema. Moderate changes were characterized by slight thickening of the alveolar walls caused by fibrin and prominent type 2 pneumocytes (type 2 pneumocyte hyperplasia), more prominent perivascular edema, and regular intra-alveolar macrophages. Severe interstitial pneumonia was characterized by diffuse hypercellularity, obscuring the small airways. Whereas the majority of animals vaccinated with IPR8+m229 had moderate lung pathology, most animals receiving IPR8-R848 exhibited mild disease. (**D**) Representative H&E-stained sections for mild, moderate, and marked changes in the lungs of challenged animals are shown. Original magnification ×20.

Critically, the enhanced immune response in the infants vaccinated with the R848-conjugated virus particle was clinically beneficial because these individuals exhibited increased virus clearance and reduced disease. The relative contribution of Ab versus T cell responses to the increased clearance is presently unknown. Given that the challenge virus was the same as that used in the vaccine, it is possible that Ab is a primary mediator.

We note that in our model, virus-specific IgG is nearly undetectable in the infants at the time of vaccination because the adult animals do not have appreciable virus-specific Ab. Admittedly, this is in contrast with what would be observed in human infants, who would have mother-derived Ab. The goal of our study was to develop vaccines that could overcome the impaired neonate immune response and induce robust adaptive immunity. It will be critical in future studies to understand the impact of pre-existing Ab on our approach and to identify strategies that can allow for immune response induction in the face of maternal Ab.

The success of R848 is likely the result of both the TLRs targeted and the linkage to the virus particle. TLR activation via R848 has been shown to be a potent activator of neonatal DCs (34), which are generally suboptimally responsive to TLR ligation (52–54). In addition, a recent study in mice reported the arrangement of R848 is a profound determinant of its in vivo activity (55). In that study, the authors tested the stimulatory capacity of HPMA-based polymers to which R848 was bound. When conditions were used in which these structures formed particles, R848 was found to possess much greater activity with regard to stimulation of innate immune responses in vivo, leading ultimately to increased $CD8^+$ and Th1 responses. The basis for the increased activity was found to be enhanced uptake by APC in the draining lymph node. Immunostimulatory particulate forms of the R848-polymer were ~700 nm in diameter compared with the poorly stimulatory unimolecular polymer coils of 10–20 nm (55). It is tempting to speculate that attachment of R848 to the influenza virion, which is ~100 nm, mimics the structure and function of the particulate forms of the R848-polymer.

An additional strategy evaluated in this study was the delivery of multiple TLR ligands. A number of studies support the increased stimulatory capacity of combined TLR (30, 36, 37, 56, 57). As an example, T cells derived from human cord blood that were stimulated with the combination of TLR2 and TLR5 agonists exhibited greater proliferation and cytokine production compared with cells stimulated in the presence of either agonist alone (58). We chose flg for the dual-adjuvant approach given our previous studies in this NHP infant model revealed an flg-mediated increase in Ab and T cell recall responses (43), although these were less robust than those we have obtained with the R848-conjugated vaccine. Surprisingly, the addition of flg to IPR8-R848 had limited benefit with regard to Ab generation and T cell number, with the exception of the trachea where animals vaccinated with the combined adjuvants showed higher levels of influenza-specific IgG pc. However, this enhanced level of Ab did not appear to provide greater protection from pulmonary damage or increased clearance. In fact, this response may have been less protective with regard to tissue damage in our model. Thus, at least in the context of our inactivated R848-congugated influenza virus vaccine, there is

limited evidence of benefit in the neonate from the combination of R848 and flg. Whether an alternative TLR ligand would result in a superior immune response and protection from pulmonary damage is a question that merits further investigation.

In summary, these data show vaccination of NHP neonates with R848-conjugated inactivated influenza virus results in an exceptionally robust Ab response after primary vaccination, boost, and challenge, as well as a potent IFN-γ–producing recall T cell response. We saw no signs of adverse reactions in infants receiving this construct; thus, there appear to be no safety concerns that would obviate further study. These data strongly support the utility of R848-conjugated inactivated influenza virus as an effective vaccine in this vulnerable population.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Foote, E. M., R. J. Singleton, R. C. Holman, S. M. Seeman, C. A. Steiner, M. Bartholomew, and T. W. Hennessy. 2015. Lower respiratory tract infection hospitalizations among American Indian/Alaska Native children and the general United States child population. *Int. J. Circumpolar Health* 74: 29256.
2. Esposito, S., and N. Principi. 2009. The rational use of influenza vaccines in healthy children and children with underlying conditions. *Curr. Opin. Infect. Dis.* 22: 244–249.
3. Groothuis, J. R., M. J. Levin, G. P. Rabalais, G. Meiklejohn, and B. A. Lauer. 1991. Immunization of high-risk infants younger than 18 months of age with split-product influenza vaccine. *Pediatrics* 87: 823–828.
4. Halasa, N. B., M. A. Gerber, Q. Chen, P. F. Wright, and K. M. Edwards. 2008. Safety and immunogenicity of trivalent inactivated influenza vaccine in infants. *J. Infect. Dis.* 197: 1448–1454.
5. Lin, J., S. Somanathan, S. Roy, R. Calcedo, and J. M. Wilson. 2010. Lung homing CTLs and their proliferation ability are important correlates of vaccine protection against influenza. *Vaccine* 28: 5669–5675.
6. Tamura, S., T. Tanimoto, and T. Kurata. 2005. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. *Jpn. J. Infect. Dis.* 58: 195–207.
7. Welliver, T. P., R. P. Garofalo, Y. Hosakote, K. H. Hintz, L. Avendano, K. Sanchez, L. Velozo, H. Jafri, S. Chavez-Bueno, P. L. Ogra, et al. 2007. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. *J. Infect. Dis.* 195: 1126–1136.
8. Eichelberger, M. C., M. L. Wang, W. Allan, R. G. Webster, and P. C. Doherty. 1991. Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. *J. Gen. Virol.* 72: 1695–1698.
9. Eichelberger, M., H. Golding, M. Hess, J. Weir, K. Subbarao, C. J. Luke, M. Friede, and D. Wood. 2008. FDA/NIH/WHO public workshop on immune correlates of protection against influenza A viruses in support of pandemic vaccine development, Bethesda, Maryland, US, December 10-11, 2007. *Vaccine* 26: 4299–4303.
10. Bungener, L., F. Geeraedts, W. Ter Veer, J. Medema, J. Wilschut, and A. Huckriede. 2008. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. *Vaccine* 26: 2350–2359.
11. Moran, T. M., H. Park, A. Fernandez-Sesma, and J. L. Schulman. 1999. Th2 responses to inactivated influenza virus can Be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. *J. Infect. Dis.* 180: 579–585.
12. Weldon, W. C., B. Z. Wang, M. P. Martin, D. G. Koutsonanos, I. Skountzou, and R. W. Compans. 2010. Enhanced immunogenicity of stabilized trimeric soluble influenza hemagglutinin. *PLoS One* 5: e12466.
13. Siegrist, C. A. 2007. The challenges of vaccine responses in early life: selected examples. *J. Comp. Pathol.* 137(Suppl. 1): S4–S9.
14. Adkins, B., C. Leclerc, and S. Marshall-Clarke. 2004. Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* 4: 553–564.
15. Zaghouani, H., C. M. Hoeman, and B. Adkins. 2009. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. *Trends Immunol.* 30: 585–591.
16. Li, L., H. H. Lee, J. J. Bell, R. K. Gregg, J. S. Ellis, A. Gessner, and H. Zaghouani. 2004. IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. *Immunity* 20: 429–440.
17. Lee, H. H., C. M. Hoeman, J. C. Hardaway, F. B. Guloglu, J. S. Ellis, R. Jain, R. Divekar, D. M. Tartar, C. L. Haymaker, and H. Zaghouani. 2008. Delayed maturation of an IL-12-producing dendritic cell subset explains the early Th2 bias in neonatal immunity. *J. Exp. Med.* 205: 2269–2280.
18. Roux, X., A. Remot, A. Petit-Camurdan, M. A. Nahori, H. Kiefer-Biasizzo, G. Marchal, M. Lagranderie, and S. Riffault. 2011. Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells. *Eur. J. Immunol.* 41: 2852–2861.
19. Naderi, N., A. A. Pourfathollah, K. Alimoghaddam, and S. M. Moazzeni. 2009. Cord blood dendritic cells prevent the differentiation of naïve T-helper cells towards Th1 irrespective of their subtype. *Clin. Exp. Med.* 9: 29–36.
20. Winkler, S., M. Willheim, K. Baier, D. Schmid, A. Aichelburg, W. Graninger, and P. G. Kremsner. 1999. Frequency of cytokine-producing T cells in patients of different age groups with *Plasmodium falciparum* malaria. *J. Infect. Dis.* 179: 209–216.
21. Xainli, J., M. Baisor, W. Kastens, M. Bockarie, J. H. Adams, and C. L. King. 2002. Age-dependent cellular immune responses to *Plasmodium* vivax Duffy binding protein in humans. *J. Immunol.* 169: 3200–3207.
22. Randolph, D. A. 2005. The neonatal adaptive immune system. *Neoreviews* 6: e454–e462.
23. Zhao, Y., Z. P. Dai, P. Lv, and X. M. Gao. 2002. Phenotypic and functional analysis of human T lymphocytes in early second- and third-trimester fetuses. *Clin. Exp. Immunol.* 129: 302–308.
24. Clerici, M., L. DePalma, E. Roilides, R. Baker, and G. M. Shearer. 1993. Analysis of T helper and antigen-presenting cell functions in cord blood and peripheral blood leukocytes from healthy children of different ages. *J. Clin. Invest.* 91: 2829–2836.
25. Miscia, S., A. Di Baldassarre, G. Sabatino, E. Bonvini, R. A. Rana, M. Vitale, V. Di Valerio, and F. A. Manzoli. 1999. Inefficient phospholipase C activation and reduced Lck expression characterize the signaling defect of umbilical cord T lymphocytes. *J. Immunol.* 163: 2416–2424.
26. Fernandez, M. A., F. K. Puttur, Y. M. Wang, W. Howden, S. I. Alexander, and C. A. Jones. 2008. T regulatory cells contribute to the attenuated primary $CD8^+$ and $CD4^+$ T cell responses to herpes simplex virus type 2 in neonatal mice. *J. Immunol.* 180: 1556–1564.
27. Holbrook, B. C., S. L. Hayward, L. K. Blevins, N. Kock, T. Aycock, G. D. Parks, and M. A. Alexander-Miller. 2015. Nonhuman primate infants have an impaired respiratory but not systemic IgG antibody response following influenza virus infection. *Virology* 476: 124–133.
28. Maisonneuve, C., S. Bertholet, D. J. Philpott, and E. De Gregorio. 2014. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. *Proc. Natl. Acad. Sci. USA* 111: 12294–12299.
29. Fujita, Y., and H. Taguchi. 2012. Overview and outlook of Toll-like receptor ligand-antigen conjugate vaccines. *Ther. Deliv.* 3: 749–760.
30. Ma, R., J. L. Du, J. Huang, and C. Y. Wu. 2007. Additive effects of CpG ODN and R-848 as adjuvants on augmenting immune responses to HBsAg vaccination. *Biochem. Biophys. Res. Commun.* 361: 537–542.
31. Wille-Reece, U., B. J. Flynn, K. Loré, R. A. Koup, R. M. Kedl, J. J. Mattapallil, W. R. Weiss, M. Roederer, and R. A. Seder. 2005. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proc. Natl. Acad. Sci. USA* 102: 15190–15194.
32. Tomai, M. A., R. L. Miller, K. E. Lipson, W. C. Kieper, I. E. Zarraga, and J. P. Vasilakos. 2007. Resiquimod and other immune response modifiers as vaccine adjuvants. *Expert Rev. Vaccines* 6: 835–847.
33. Vasilakos, J. P., and M. A. Tomai. 2013. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. *Expert Rev. Vaccines* 12: 809–819.
34. Levy, O., E. E. Suter, R. L. Miller, and M. R. Wessels. 2006. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. *Blood* 108: 1284–1290.
35. Peng, G., Z. Guo, Y. Kiniwa, K. S. Voo, W. Peng, T. Fu, D. Y. Wang, Y. Li, H. Y. Wang, and R. F. Wang. 2005. Toll-like receptor 8-mediated reversal of $CD4^+$ regulatory T cell function. *Science* 309: 1380–1384.
36. Warger, T., P. Osterloh, G. Rechtsteiner, M. Fassbender, V. Heib, B. Schmid, E. Schmitt, H. Schild, and M. P. Radsak. 2006. Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. *Blood* 108: 544–550.
37. Krumbiegel, D., F. Zepp, and C. U. Meyer. 2007. Combined Toll-like receptor agonists synergistically increase production of inflammatory cytokines in human neonatal dendritic cells. *Hum. Immunol.* 68: 813–822.
38. Klimov, A., A. Balish, V. Veguilla, H. Sun, J. Schiffer, X. Lu, J. M. Katz, and K. Hancock. 2012. Influenza virus titration, antigenic characterization, and serological methods for antibody detection. *Methods Mol. Biol.* 865: 25–51.
39. McDermott, P. F., F. Ciacci-Woolwine, J. A. Snipes, and S. B. Mizel. 2000. High-affinity interaction between gram-negative flagellin and a cell surface polypeptide results in human monocyte activation. *Infect. Immun.* 68: 5525–5529.
40. Weimer, E. T., S. E. Ervin, D. J. Wozniak, and S. B. Mizel. 2009. Immunization of young African green monkeys with OprF epitope 8-OprI-type A- and B-flagellin fusion proteins promotes the production of protective antibodies against nonmucoid *Pseudomonas aeruginosa*. *Vaccine* 27: 6762–6769.
41. Honko, A. N., and S. B. Mizel. 2004. Mucosal administration of flagellin induces innate immunity in the mouse lung. *Infect. Immun.* 72: 6676–6679.
42. Manicassamy, B., S. Manicassamy, A. Belicha-Villanueva, G. Pisanelli, B. Pulendran, and A. García-Sastre. 2010. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. *Proc. Natl. Acad. Sci. USA* 107: 11531–11536.

43. Kim, J. R., B. C. Holbrook, S. L. Hayward, L. K. Blevins, M. J. Jorgensen, N. D. Kock, K. De Paris, R. B. D'Agostino, Jr., S. T. Aycock, S. B. Mizel, et al. 2015. Inclusion of flagellin during vaccination against influenza enhances recall responses in nonhuman primate neonates. *J. Virol.* 89: 7291–7303.
44. Ketloy, C., A. Engering, U. Srichairatanakul, A. Limsalakpetch, K. Yongvanitchit, S. Pichyangkul, and K. Ruxrungtham. 2008. Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates. *Vet. Immunol. Immunopathol.* 125: 18–30.
45. Turley, C. B., R. E. Rupp, C. Johnson, D. N. Taylor, J. Wolfson, L. Tussey, U. Kavita, L. Stanberry, and A. Shaw. 2011. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. *Vaccine* 29: 5145–5152.
46. Taylor, D. N., J. J. Treanor, C. Strout, C. Johnson, T. Fitzgerald, U. Kavita, K. Ozer, L. Tussey, and A. Shaw. 2011. Induction of a potent immune response in the elderly using the TLR-5 agonist, flagellin, with a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125, STF2.HA1 SI). *Vaccine* 29: 4897–4902.
47. Caron, G., D. Duluc, I. Frémaux, P. Jeannin, C. David, H. Gascan, and Y. Delneste. 2005. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. *J. Immunol.* 175: 1551–1557.
48. Bekeredjian-Ding, I. B., M. Wagner, V. Hornung, T. Giese, M. Schnurr, S. Endres, and G. Hartmann. 2005. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *J. Immunol.* 174: 4043–4050.
49. Bourke, E., D. Bosisio, J. Golay, N. Polentarutti, and A. Mantovani. 2003. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. *Blood* 102: 956–963.
50. Kabelitz, D. 2007. Expression and function of Toll-like receptors in T lymphocytes. *Curr. Opin. Immunol.* 19: 39–45.
51. Wang, G., Y. Miyahara, Z. Guo, M. Khattar, S. M. Stepkowski, and W. Chen. 2010. "Default" generation of neonatal regulatory T cells. *J. Immunol.* 185: 71–78.
52. De Wit, D., S. Tonon, V. Olislagers, S. Goriely, M. Boutriaux, M. Goldman, and F. Willems. 2003. Impaired responses to toll-like receptor 4 and toll-like receptor 3 ligands in human cord blood. *J. Autoimmun.* 21: 277–281.
53. Levy, O., M. Coughlin, B. N. Cronstein, R. M. Roy, A. Desai, and M. R. Wessels. 2006. The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. *J. Immunol.* 177: 1956–1966.
54. Kollmann, T. R., J. Crabtree, A. Rein-Weston, D. Blimkie, F. Thommai, X. Y. Wang, P. M. Lavoie, J. Furlong, E. S. Fortuno, III, A. M. Hajjar, et al. 2009. Neonatal innate TLR-mediated responses are distinct from those of adults. *J. Immunol.* 183: 7150–7160.
55. Lynn, G. M., R. Laga, P. A. Darrah, A. S. Ishizuka, A. J. Balaci, A. E. Dulcey, M. Pechar, R. Pola, M. Y. Gerner, A. Yamamoto, et al. 2015. In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity. *Nat. Biotechnol.* 33: 1201–1210.
56. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* 7: 179–190.
57. Zhu, Q., C. Egelston, S. Gagnon, Y. Sui, I. M. Belyakov, D. M. Klinman, and J. A. Berzofsky. 2010. Using 3 TLR ligands as a combination adjuvant induces qualitative changes in T cell responses needed for antiviral protection in mice. *J. Clin. Invest.* 120: 607–616.
58. McCarron, M., and D. J. Reen. 2009. Activated human neonatal CD8+ T cells are subject to immunomodulation by direct TLR2 or TLR5 stimulation. *J. Immunol.* 182: 55–62.