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Adjuvanting an inactivated influenza vaccine with flagellin improves the function and quantity of the long-term antibody response in a nonhuman primate neonate model

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Abstract

Young infants are at significantly increased risk of developing severe disease following infection with influenza virus. At present there is no approved vaccine for individuals below the age of six months given previous studies showing a failure of these individuals to efficiently seroconvert. Given the major impact of influenza on infant health, it is critical that we develop vaccines that will be safe and effective in this population. Using a nonhuman primate (NHP) model, we have evaluated the ability of an inactivated influenza virus vaccine adjuvanted with flagellin to result in long term immune responses in neonates. To evaluate this critical attribute, neonate NHP were vaccinated and boosted with inactivated influenza virus in combination with either flagellin or a mutant inactive flagellin control. Our studies show that inclusion of flagellin resulted in a significant increase (5-fold, $p=0.04$) in influenza virus-specific IgG antibody at 6 months post-vaccination. In addition, the antibody present at this late time was of higher affinity (2.4-fold, $p=0.02$). Finally a greater percentage of infants had detectable neutralizing antibody. These results support the use of flagellin in neonates as an adjuvant that promotes long-lived, high affinity antibody responses.

Keywords

vaccine; influenza virus; antibody; affinity; T lymphocyte; neonate; nonhuman primate

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1. INTRODUCTION

Infection with influenza virus poses a serious health risk in young infants. In a cohort of US children, 33% developed influenza virus infection within the first year of life [1]. In addition to the heightened susceptibility to infection, infants less than 6 months of age are more vulnerable to the development of severe disease [1]. Analysis of children under the age of 5 years who were hospitalized as a result of influenza revealed that nearly half were under the age of 6 months [2].

It is now recommended that pregnant women receive the inactivated influenza vaccine, which allows passive transfer of maternal antibody that can increase protection in the newborn. However, in spite of the known benefits of vaccination, many pregnant women are reluctant to receive the vaccine due to safety concerns [3, 4]. Thus, significant challenges remain in the protection of infants through this approach. The alternative is vaccination of the infant. However, at present there is no approved vaccine for infants less than 6 months of age. A principal factor in the lack of approval for use of these vaccines in very young infants is the reported poor immunogenicity [5, 6]. Previous studies have shown that a single dose of the trivalent influenza vaccine (TIV) fails to induce seroconversion against H1N1 strains in infants less than 3–5 months of age [5]. A second dose resulted in protective titer in approximately 29% of individuals [5]. Not surprisingly, a correlation was observed between age and the rate of conversion with older infants converting at a higher rate than younger infants [5].

The immune system of very young infants often responds poorly, even following infection [7, 8]. Dendritic cell (DC) maturation is a critical regulator of adaptive immune responses, necessary for appropriate T cell for proliferation, survival, and the acquisition of effector function. Multiple studies have reported the decreased ability of DC and monocytes isolated from the cord blood of human neonates to respond to danger signals, i.e. Toll like receptor (TLR) agonists, compared to DC isolated from adults [9–12]. In addition to the impaired responsiveness of DC, T lymphocytes from neonates exhibit inherent defects in their ability to undergo activation and differentiation [13–15]. This is in part the consequence of diminished activation of multiple molecules involved in T cell receptor signaling [16, 17].

In addition to the above, impaired accessory cells (T follicular helper (T_{FH}) and follicular DC) [18, 19] and inherent defects in B cell survival and differentiation [20] likely contribute to the diminished antibody responses often reported in infants [7, 8, 21]. Reduced expression of B cell maturation antigen (BCMA) and B cell activating factor receptor (BAFF-R) may in part explain reduced B cell survival and differentiation, while survival of plasmablasts and generation of long-lived antibody secreting cells is likely hampered by decreased levels of a proliferation inducing ligand (APRIL) [22]. Depressed antibody responses are prolonged in infants, with IgG production generally weak for the first year of life [23, 24].

The development of successful vaccine approaches for neonates requires identification of signals that can overcome the reduced/inappropriate adaptive immune responses in these individuals. TLR agonists have received a great deal of attention as potential adjuvants. Here we have evaluated the capacity of flagellin (flg), a TLR5 agonist, to overcome the

deficiencies associated with the neonatal response. Flagellin has proven to be a potent adjuvant for the induction of antibody responses in a number of experimental animal models (for review see [25]). Rationale to support the use of flagellin specifically in the context of influenza vaccination comes from studies in mice demonstrating its inclusion in an inactivated influenza virus vaccine resulted in increased antibody titers and protection [26]. The potency of flagellin as an adjuvant is in part due to its ability to induce activation of DC [27]. In addition, TLR5 agonists can act directly on primate T cells [25, 28, 29]. Critically, there are data supporting the effectiveness of this adjuvant in promoting activation of T cells from neonates [30]. Finally, a recent study using a mouse model reported TLR5 expression on activated B cells and plasmablasts [31]. As a result, flagellin can directly promote antibody responses. Thus, this adjuvant has the potential to facilitate the generation of an immune response in neonates through its action on multiple immune cells.

For our studies we have utilized an infant nonhuman primate model. This was critical to our analysis as the distribution and/or function of TLR5 in primates can differ from mice [27, 32]. For example, flagellin does not directly stimulate murine T cells [32, 33]. An additional critical aspect of this model is the significantly longer period of infancy in NHP, which allows appropriate assessment of boosting strategies. Thus, the primate model offers many advantages for the optimal assessment of the action of flagellin in neonates.

We have previously explored the acute response induced by an inactivated influenza virus A/Puerto Rico/8/1934 (IPR8) vaccine adjuvanted with flg following administration to neonate African green monkeys (AGM) [34]. Nursery reared animals were vaccinated at 4–6 days of age and boosted 21 days later. Challenge was performed at 3.5–4 weeks following boost. Animals that received flagellin adjuvanted vaccine exhibited a significantly increased antibody response following boost and challenge. In addition, they exhibited an enhanced T cell response compared to their non-adjuvanted vaccine counterparts [34]. These results suggested flagellin has utility as an adjuvant in neonates.

However, in addition to eliciting a robust immune response, an efficacious vaccine should result in long-lived memory. The aim of the current study was to evaluate the ability of the flagellin adjuvanted inactivated influenza vaccine to promote a long-lived IgG and IgM antibody responses in an independent group of infant animals (as those in our initial study were necropsied following challenge). We assessed IgG and IgM responses as both are reported to contribute to neutralization of influenza virus [35, 36]. The results presented here demonstrate increased influenza-virus specific IgG, although not IgM, responses at 6 months following vaccination in infants administered IPR8+flagellin compared to those that received IPR8 containing an inactive form of flagellin (m229). In addition, the antibody had improved function as measured by affinity. Thus, flagellin results in an increase in both the function and quantity of the memory response elicited in neonates following vaccination.

2. MATERIALS AND METHODS

2.1 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. All animal protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine.

2.2 Vaccination

A/Puerto Rico/8/1934 [H1N1] (PR8) virus stocks were grown and titered (egg infectious dose (EID₅₀)) in fertilized chicken eggs essentially as described previously [37]. Stocks were diluted in PBS, flash frozen, and stored at -80°C. Inactivation of IPR8 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. Flagellin from *Salmonella enteritidis* was prepared as previously described [38]. At 1–6 days of age, infants were vaccinated with 45µg of inactivated virus (IPR8) in the presence of 10µg of flagellin (flg) or inactive 229 mutant flagellin (m229) (Fig. 1). All injections were delivered intramuscularly in the deltoid muscle (500µl volume). Animals were boosted 21 days later. Five infants received the m229 adjuvanted vaccine (4 males and 1 female) and 6 received the flagellin adjuvanted vaccine (3 males and 3 females). For negative controls, one infant received PBS (female) and three were untreated animals 6 months of age (1 male and 2 females). Antibody levels were similar in the non-vaccinated and PBS animals. Blood was drawn on days 1, 10, and 21 following initial vaccination and days 10 and 21 and approximately 3 months (d86–116) and 6 months (d168–203) post boost (Fig. 1).

2.3 ELISA for the detection and affinity determination of influenza virus-specific antibody

Detection of virus-specific IgG and IgM antibody was performed as previously reported [34]. Threshold titer was defined as the value that reached three times the assay background, i.e. wells that received only sample diluent. For determination of relative affinity, binding was disrupted by incubation with titrated concentrations (two-fold dilutions ranging from 5M-0.078M) of sodium isothiocyanate for 15 minutes at room temperature prior to addition of HRP-conjugated anti-monkey IgG. The remaining steps for development of the assay are as in [34].

2.4 Neutralization assay

Neutralization capacity was assessed as previously reported [34]. Briefly heat-inactivated plasma samples were serially diluted and mixed with PR8-GFP (kindly provided by Dr. Adolfo Garcia-Sastre [39]). U937 cells were then added to each well and incubated overnight at 37°C. The percentage of U937 cells that were positive for GFP was quantified by flow cytometry. The dilution at which the 50% maximum PR8-GFP infected U937 cells occurred was calculated by nonlinear regression (Graphpad Prism). Samples wherein 50% inhibition was not reached at the highest concentration of plasma were assigned a value of 0.

2.5 T cell ELISPOT

IFN-γ-producing cells were quantified following culture with bone marrow derived dendritic cells that had been infected with GFP-PR8 virus or mock infected as previously reported [34]. Spots were analyzed by ImmunoSpot Analyzer (Cellular Technology Ltd) and ImmunoSpot (version 3.2) software. The number of cytokine secreting cells per tissue was calculated based on the total number of cells recovered.

2.6 Statistical analysis

For continuous outcomes, groups were compared using 2-sample t-tests (if the treatment group had two levels) or analysis of variance (ANOVA) models (if there were 3 or more levels for the treatment group). If outcome data were not normally distributed logarithmic transformations were used prior analyses. For analyses that included repeated measures, a 2-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. Data were analyzed using Prism 5 software or SAS Version 9.3.

3 RESULTS

3.1 Adjuvanting inactivated influenza vaccine with flagellin prolongs virus-specific IgG responses

In this study, we determined whether the presence of flagellin in an inactivated influenza virus vaccine could promote an elevated, sustained antibody response in young infants. To address this question, infant AGM were vaccinated at 1–6 days of age and boosted 21 days later with an inactivated influenza vaccine (IPR8) containing flg or the inactive flg construct (m229) (Fig. 1). Each vaccine group had a similar age distribution at the time of initial vaccination and contained male and female animals. Control infants received PBS or were unvaccinated. Infants remained with their mothers throughout the 6 month course of the study.

Influenza virus-specific antibody responses were measured at d10 and 21 post vaccination/boost as well as at 3 and 6 months post boost. Analysis of the memory responses showed that on average the geometric mean influenza virus-specific IgG antibody titer was higher at 3 months post vaccination (4,526 vs. 1,600) in infants receiving the IPR8+flg vaccine (Fig. 2A). At six months post vaccination there was a significantly higher antibody titer in IPR8+flg vaccinated versus IPR8+m229 vaccinated animals (1,056 vs. 200) (Fig. 2A). We did not observe significant differences in IgM antibody between animals vaccinated with m229 vs. flagellin (Fig. 2B). We considered whether there might be gender related differences in responsiveness. While we found none, detection would have required very large differences given the low number of animals of each gender within the group. Together these data show inclusion of flg results in a higher level of virus-specific IgG antibody in infants at 6 months following vaccination.

3.2 Analysis of neutralizing antibody present at 6 months post vaccination in infants receiving inactivated influenza vaccine adjuvanted with flagellin versus m229

We next assessed the level of neutralizing antibody present in the infants following vaccination as determined by the ability of plasma from immunized animals to inhibit infection of tissue culture cells with a green fluorescent protein (GFP)-expressing influenza virus [40]. Using this approach we determined the half maximal inhibitory concentration (IC₅₀) defined as the dilution of plasma that resulted in 50% inhibition of infectivity. This approach was chosen as it is a direct measure of the ability of the antibody to prevent infection. It is clear from the data that the level of neutralizing antibody can vary greatly among animals within each vaccine group (Fig. 3). This variability resulted in group

differences that did not reach statistical significance for our sample size. However, there was a trend towards higher neutralizing antibody throughout the 6 month period of the study. In addition, importantly 2 of the 5 infants immunized with IPR8+m229 had undetectable neutralizing antibody responses at 6 months, whereas all animals in the flagellin-adjuvanted group retained neutralizing antibody that was well above the limit of assay detection at 6 months, with 2 animals having high levels (IC_{50} value of 37 and 49). Assessment using the standard hemagglutination inhibition assay (HAI) yielded values of 40 and 160, respectively (data not shown), which is considered to be protective [41]. Using our PR8-GFP based assay, the highest titers for the IPR8+m229 infants at this timepoint were 4.4 and 2.3 (Fig. 3). None of the IPR8+m229 infants had detectable neutralizing titers by HAI (data not shown).

3.3 Inactivated influenza vaccine adjuvanted with flagellin results in an increase in the affinity of influenza virus-specific IgG antibody present at 6 months following boost

We next asked whether the presence of flagellin during vaccination resulted in an increase in the affinity of the influenza virus-specific IgG present at six months. Affinity was assessed by determining the ability of sodium isothiocyanate to disrupt binding [42]. For this analysis we chose dilutions of plasma that allowed for similar amounts of total virus-specific IgG to obviate any potential for apparent increases in affinity to be the result of differences in the overall amount of antibody available for binding. This analysis revealed a significant increase in affinity as approximately 2.4-fold more NaSCN was required to disrupt 50% of the antibody binding in infants receiving the flagellin adjuvanted vaccine (Fig. 4).

3.4 Analysis of IFN γ -producing, influenza virus specific T cells following vaccination with inactivated influenza virus adjuvanted with flg or m229

An important goal of influenza vaccination is the generation of a long-lived T cells that are capable of producing IFN γ in response to viral challenge. Thus, the presence of IFN γ producing cells was assessed at six months following vaccination. Three animals from each group were necropsied for these analyses. Cells from the draining axillary lymph node of infants vaccinated with IPR8+m229 or IPR8+flg were stimulated by culture in the presence of influenza virus infected autologous dendritic cells. While IFN γ -producing influenza-specific T cells were detected in both vaccinated groups, there was no significant difference in the animals vaccinated with the flagellin adjuvanted versus m229-adjuvanted IPR8 construct (Fig. 5). Thus, inclusion of flagellin in vaccines for neonates based on inactivated influenza virus may not be an effective approach to promote an increase in memory T cell responses.

4 DISCUSSION

The goal of vaccination is to induce a long-lived immune response that can protect from/limit infection following pathogen encounter. Our previous study of short term immune responses generated by vaccination with an inactivated influenza virus vaccine demonstrated a beneficial adjuvant effect of flagellin in increasing recall responses in neonates following virus challenge [34]. In the current study we extended these observations by exploring the ability of the immune responses induced by inactivated influenza virus adjuvanted with

flagellin to be maintained over a six month period. Our results show that the presence of flagellin resulted in a qualitative and quantitative increase in influenza virus-specific IgG antibody at 6 months post-vaccination.

We acknowledge that while the results are promising, the study does have limitations. For example, the number of infants vaccinated was small. In addition, the mothers were influenza naïve and thus had no or very low IgG antibody capable of recognizing influenza virus (average titer of 120 ± 1.6). This was reflected in the infants, who also had minimal or undetectable IgG antibody against influenza virus. This is in contrast to human infants, where pre-existing antibody would be expected as a result of passively acquired maternal antibody. It is likely that the range of antibody among infants would differ considerably, as there is notable variation among pregnant women in the level of antibody present [43]. Understanding vaccination strategies that can work in the face of maternal antibody is a critical next step in the development of vaccine approaches that can be effective in infants.

The adjuvant potential of flagellin has been explored in a number of vaccine constructs including admixed with a soluble protein (e.g. [34, 44]), fused/conjugated to proteins (e.g. [45–47]), incorporated into virus like particles [48, 49] and expressed from a plasmid [50]. Flagellin has been shown to increase antibody as well as T cell responses (for review see [25, 51]). Based on these promising results, adult-targeted flagellin containing vaccines against plague, influenza, and campylobacter-mediated diarrhea have completed phase 1 clinical trials. With regard to influenza, promising results have been reported for vaccine constructs comprised of flagellin fused to the globular head domain of the influenza hemagglutinin (HA) antigen [47, 52] and flagellin fused to the ectodomain of the highly conserved influenza matrix protein 2 (M2e) [53]. Flagellin fusion to the globular head of HA has been explored as a vaccine in the hyporesponsive elderly population, with results showing enhanced immunogenicity in this difficult to vaccinate cohort [47].

Neonates pose a challenge with regard to both elicitation of acute antibody responses as well as the generation of long lived immunity [54]. In the current study, we report enhanced durability of antibody responses with flagellin-adjuvanted vaccine relative to non-adjuvanted vaccine in a neonate AGM model. One explanation for the higher level of antibody observed at 6 months in infants vaccinated in the presence of flagellin is the overall increase in the level of antibody produced in the acute response to vaccination [34]. However, we do not think this solely accounts for the effect, as the increase in virus-specific IgG in IPR8+flg vs. IPR8+m229 vaccinated infants at d21 post boost is less than that observed at 6 months (2.5 vs. 5.3), suggesting a greater maintenance of systemic influenza-specific IgG antibody in the flg group. Our analysis of antibody secreting cells in the bone marrow at 6 months found these cells were not readily detectable (data not shown); thus, whether the increased antibody is due to an increased number of plasma cells or a higher production of antibody on a per cell basis is not known.

The failure to maintain antibody in neonates has been attributed at least in part to the inability to efficiently maintain plasma cells. A critical contributor to this may be the reduced availability of macrophage derived APRIL in the bone marrow, as has been reported in neonatal mice [22]. TLR5 expression on macrophages raises the possibility that the

flagellin may provide signals that increase the production of APRIL during a critical window of plasma cell differentiation. Given that the persistence of flagellin *in vivo* is likely limited and the recent demonstration of TLR5 expression on plasmablasts/short-lived plasma cells [31], we would speculate that the modulating flagellin signal comes during the boost as plasmablasts are developing. The increased presence of APRIL during this critical window of differentiation may allow prolonged survival of plasma cells. While IL-21 is known to provide a potent signal for the differentiation of plasma cells (for review see [55]), data suggest *in vivo* administration of flagellin does not promote IL-21 production in lymphoid tissues [56], making modulation of IL-21 a less likely mechanism the ability of flagellin to sustain antibody levels in the vaccinated infants.

Another critical aspect of flagellin revealed by our study is its ability to induce higher affinity antibody. Analysis of infants at 6 months post-vaccination identified a 2.4 increase in the amount of NaSCN required to disrupt antibody binding. Generation of high affinity antibody in the context of the neonate has long been considered a major challenge [23, 24]. In humans, somatic hypermutation is rare below the age of 6 months and when it occurs there is limited evidence of selection [57, 58]. Thus, the ability of flagellin to increase affinity in the very young infants utilized in our study is striking. An appealing possibility is that this is the result of modulation of T_{FH} responses, which are reported to be impaired in infants [21]. Flagellin has the potential to act through both maturation of DC involved in activation of T_{FH} as well as directly on the T cell through its expression of TLR5 [25, 30].

The similar number of IFN γ -producing T cells at 6 months post vaccination was unexpected given the increase we observed following virus challenge in animals receiving flagellin adjuvanted versus non-adjuvanted vaccine [34]. This previous observation would have predicted an increase in the memory pool in infants that received flagellin. However, it is important to note that our previous study assessed the response following virus challenge and as such the number of cells that were generated by vaccination is unknown. One possibility is that the quality, as opposed to the quantity, of the cells was modulated by vaccination in a manner that promoted more efficient activation and expansion following virus infection. Another important consideration is the difference in the environment in which our previous challenge study versus the memory study described here was performed. Challenged animals were nursery-reared as opposed to the mother-reared infants evaluated in the current study. If and how this difference may impact T cells is unknown, but certainly merits further investigation. In summary, these data provide the first evidence for the ability of flagellin to 1) promote long-lived antibody responses and 2) increase the affinity of antibody following vaccination of neonates. Understanding how flagellin drives these critical aspects of protective immune responses in infants will be an important area for future study.

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HIGHLIGHTS

- Neonate influenza vaccines including flagellin promote increased virus-specific IgG at 6 months
- Neonate vaccines including flagellin promote higher affinity virus-specific IgG at 6 months
- Addition of flagellin does not increase the number of IFN γ producing memory cells

Figure 1. Schematic of vaccination and sampling strategy

Infants were vaccinated at 1–6 days of age. Twenty-one days later, infants received a second vaccination. Blood (B) samples were obtained at d10 and 21 post vaccination/boost and again at approximately 3 months (d86–116) and 6 months (d168–203) post boost.

Figure 2. Influenza-specific IgG in infants vaccinated with IPR8+flg versus IPR8+m229
 PR8-specific IgG (A) or IgM (B) was measured in the plasma of vaccinated (IPR8+flg n=6, IPR8+m229 n=5) and non-vaccinated infants (n=3). Data for control animals that did not receive vaccine are shown for the 6 month timepoint. A representative group of non-vaccinated infants (3–7 days of age) are shown as an indicator of baseline antibody in neonates (neo). One animal in the IPR8+flg group died at 5 months of age from infection not related to vaccination. As such there are 5 animals at the 6 month timepoint. Individual

animals and geometric means are shown. * $p < 0.05$, ** $p < 0.005$. BLD=below limit of detection.

Figure 3. Neutralizing antibody in infants vaccinated with IPR8+flg versus IPR8+m229
 Neutralizing antibody titers in plasma were measured over the course of vaccination by assessing inhibition of the ability of a GFP expressing PR8 virus to infect U937 cells. Neutralization titer was defined as the half maximal inhibitory concentration (IC_{50}), i.e. the dilution factor at which 50% infectivity was blocked. Individual animals and geometric means are shown.

Figure 4. Affinity of influenza-specific IgG antibody present at 6 months following vaccination with IPR8+flg or IPR8+m229

Titration concentrations of sodium isothiocyanate were added following binding of PR8-specific antibody in our standard ELISA protocol. Disruption of antibody binding served as a measure of affinity. Control animals that did not receive vaccine had no detectable virus-specific IgG and as such these data are not shown. Data shown represent the average of the amount of NaSCN required to reduce the OD by 50%. * $p < 0.05$

Figure 5. The presence of IFN γ -producing, influenza virus-specific T cells at 6 months following vaccination with IPR8+flg or IPR8+m229

IFN γ -producing influenza-specific T cells in the vaccine-draining axillary LN were quantified by ELISPOT at the 6 month timepoint following vaccination. The three animals sent to necropsy were included in each group.