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A Single Immunization with Inactivated H1N1 Influenza Vaccine Formulated With Delta Inulin Adjuvant (Advax™) Overcomes Pregnancy-Associated Immune Suppression and Enhances Passive Neonatal Protection

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Abstract

Pregnant women and neonates represent high-risk groups for influenza infection, and in general have suppressed responses to standard influenza vaccines due to pregnancy-associated immune suppression and immune system immaturity, respectively. We therefore wished to test whether addition of Advax™, a polysaccharide adjuvant based on delta inulin, to an inactivated influenza vaccine (A/H1N1/PR8) administered during pregnancy would safely enhance vaccine immunogenicity and thereby provide improved protection of pregnant mothers and also potentially their newborns. Pregnant mice received a single intramuscular injection of β -propiolactone inactivated H1N1 antigen alone or with Advax adjuvant. Pregnant dams receiving Advax adjuvanted vaccine exhibited significantly increased serum and breast milk anti-influenza IgG titers. This translated into higher serum anti-influenza IgG titers in the pups of these dams. Complete protection was seen in pups of dams that received Advax-adjuvanted vaccine whereas no survival was seen in pups of control mothers or mothers immunized with unadjuvanted vaccine. Cross-fostering studies confirmed that enhanced protection of pups of dams that received Advax-adjuvanted vaccine was mediated by enhanced transfer of maternal IgG to the pups via breast-feeding. The delta inulin adjuvant was not associated with any reproductive or developmental adverse effects. This study shows that Advax adjuvant was safe when administered with influenza vaccine during pregnancy and provided protection of pups not seen with administration of unadjuvanted vaccine, via enhanced breast milk transfer of anti-influenza antibodies.

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POTENTIAL CONFLICTS OF INTEREST

YH, AK, LL and NP are employees of Vaxine Pty Ltd (Adelaide Australia), which holds proprietary interests in delta inulin adjuvant technology and the Advax™ trademark.

Keywords

vaccine; influenza; pregnancy; neonate; adjuvant; immunity; breast feeding

INTRODUCTION

During the 2009 A/H1N1pdm09 influenza pandemic, as seen in previous epidemics and pandemics, pregnant women were amongst the high risk groups that suffered from increased risk of complications and mortality [1]. The World Health Organization recommended that all pregnant women should be immunized with H1N1pdm09 vaccine regardless of trimester of pregnancy. Just a single dose of H1N1pdm09 vaccine was found sufficient to induce seroprotection in most subjects. By contrast, vaccines against pandemic viruses such as H5N1 are typically poorly immunogenic and require multiple immunizations with high antigen doses and/or addition of adjuvants to achieve seroprotection [2]. This poor immunogenicity is compounded by pregnancy being a state of relative immune suppression, such that immunization during pregnancy may be less effective [3]. Inactivated seasonal influenza vaccines provide only 30–80% protection in healthy non-pregnant subjects [2] with vaccine effectiveness in pregnant women estimated to range from no protection up to 70% protection [4]. There is thus a priority to develop influenza vaccines able to overcome pregnancy-associated immune suppression. However, any vaccine used in pregnancy must also be safe for both mother and child. This is particularly relevant after reports of narcolepsy in children receiving a squalene-adjuvanted H1N1pdm09 vaccine [5].

It is also a priority to better protect newborns against influenza. Both inactivated and live influenza vaccines are not currently indicated for children below 6 months [6], despite this being a highly susceptible population. Maternal immunization is a valuable method to provide passive immunity to newborns [7]. Immunization of mothers has been shown in animal models to provide enhanced passive protection to their offspring [8, 9] and in humans has been estimated at 41–91% effective in protecting newborns against influenza [4]. Thus an enhanced potency influenza vaccine might not just better protect an immunized mother but also her future offspring.

Advax™ (Vaxine Pty Ltd, Adelaide, Australia) is a novel polysaccharide adjuvant derived from crystalline particles of polyfructofuranosyl-D-glucose (delta inulin) [10]. Advax has been shown to enhance immunogenicity and protection of vaccines against influenza [11], Japanese encephalitis [12, 13], West Nile virus [14], hepatitis B virus [15], human immunodeficiency virus [16], H5N1 influenza [17] and anthrax [18]. Advax also showed efficacy and safety when administered with a recombinant pandemic H1N1pdm09 influenza vaccine to non-pregnant adult human subjects [19].

Given its ability to broadly enhance vaccine immunogenicity, we asked whether Advax adjuvant was safe to administer during pregnancy and, if so, whether it could help overcome pregnancy-associated immunosuppression. We further asked whether administration of an Advax-adjuvanted influenza vaccine during pregnancy could provide improved passive protection to the babies. Lastly, we sought to identify the mechanism for enhanced protection of pups of mothers immunized with Advax-adjuvanted vaccine.

MATERIALS AND METHODS

Mice and Immunization Procedure

Virgin and time-mated BALB/c mice were supplied by the Flinders University central animal facility. Time-mated mice were randomized into two groups to be immunized at gestational day 7 with a single intramuscular (i.m.) injection in the hind thigh with β -propiolactone (BPL)-inactivated influenza A/Puerto Rico/08/34 (iPR8) virus antigen (Advanced Biotechnologies Inc., Columbia, MD, USA) with or without Advax™ adjuvant (Vaxine Pty Ltd, Adelaide, Australia). All procedures were performed in accordance with the Animal Experimentation Guidelines of the National Health and Medical Research Council of Australia and approved by the Flinders University Animal Welfare Committee.

Sample collection

Blood samples were collected by cheek vein bleeding. To collect breast milk, mothers were anesthetized on day 10 after birth and 0.5 IU of oxytocin (Sandoz Pty Ltd, Australia) was given i.m. 5 minutes before milking. Milk was separated by centrifugation ($4,000 \times g$, 4°C , 15 min), the top lipid layer discarded and the whey stored at -20°C until use.

Cross-fostering studies

To study the mechanism whereby neonates acquired protection against influenza from their mothers, on the day of birth pups of immunized dams were removed from their biological mother and placed with a lactating sham saline-immunized dam whose own pups had similarly been removed and placed with a lactating immunized dam. At the time of weaning, IgG titers were measured in sera of each set of pups prior to challenge at 4 weeks of age.

Influenza Challenges

The virus used for challenge experiments was a mouse-adapted H1N1/PR8 virus propagated in allantoic fluid of 10-day old embryonated hen's eggs and purified by sucrose density gradient ultracentrifugation and stored at -80°C until used. Mice were infected by intranasal administration of 50 μl (adults) or 30 μl (neonates) of PR8 virus. The 50% mouse lethal dose (LD_{50}) was estimated in adult BALB/c mice by the Reed-Muench method [20]. One adult LD_{50} corresponded to 1250 TCID_{50} on MDCK cells and the standard adult mice challenge dose we used was $8 \times \text{LD}_{50}$ with this dose giving 100% lethality in unimmunized or sham-immunized mice. Due to the increased susceptibility of neonates to influenza infection, a 5000 TCID_{50} dose ($4 \times \text{LD}_{50}$ for adult mice) was used with the virus being approximately 2 times more potent in neonatal mice. This dose induced 100% lethality in unimmunized or sham-immunized 4-week-old mice, similar to the 100% mortality seen in other studies in naïve 2-week-old mice administered a $3 \times \text{LD}_{50}$ dose of PR8 virus [8]. For challenge studies, unimmunized control groups were routinely included to ensure that the challenge dose was completely lethal. A sickness scoring system based on coat condition, posture and activity was used to assess the extent of clinical disease. Mice were evaluated daily and scored for individual symptoms. Ruffled fur (absent = 0; slightly present = 1; present = 2), hunched back (absent = 0; slightly present = 1; present = 2) and activity (normal = 0; reduced = 1; severely reduced = 2) were evaluated. The final score was the addition of each individual

symptom score (e.g. an animal showing slightly ruffled fur (1), slightly hunched back (1) and reduced activity (1) was scored as 3). Mice were euthanized if they became moribund or developed a clinical score of 6.

Antibody assays

Influenza virus specific antibodies were determined by ELISA, as previously described [11]. Briefly, 96-well ELISA plates were coated with iPR8 antigen, blocked for 1 hour, samples added for 2 hours, washed and biotinylated anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM (Abcam) plus HRP-conjugated Streptavidin was added for 1 hour, washed, TMB substrate added for 10 minutes then stopped with 1M Phosphoric Acid. Optical density was read at 450nm (OD_{450nm}) with a spectrophotometer plate reader (VersaMax, Molecular Device.) and analyzed using SoftMax Pro Software.

Quantitation of neonatal Fc receptor (FcRn)

Proximal duodenum tissue was lysed in TRI-reagent by using Mini-Beadbeater-16 (BioSpec Products, Bartlesville, OK, USA) for RNA extraction. Reverse transcription and qPCR were carried out by using iScript™ cDNA Synthesis kit (Bio-Rad) and iTaq SYBR Green Supermix with ROX (Bio-Rad) per product manuals, respectively. Primers for mouse FcRn are Fwd 5'-AACGTGGAGATGAGCACCAT-3' and Rev 5'-CGATTCCAACCACAGGCACA-3'; primers for mouse 18S are Fwd 5'-AGTTAGCATGCCAGAGTCTCG-3' and Rev 5'-TGCATGGCCGTTCTTAGTTG-3'. 2⁻ C_q method was used for expression analysis.

Statistical analysis

Mann-Whitney test was performed using GraphPad Prism version 5.01 for Windows. For survival data, curves were created using the Kaplan-Meier method and statistical analyses performed using a log-rank (Mantel-Cox) test. For all comparisons, $p < 0.05$ was considered as significant.

RESULTS

A single dose of delta inulin-adjuvanted influenza vaccine is able to protect virgin female mice

In previously published studies, two immunizations 3–4 weeks apart of delta inulin-adjuvanted inactivated PR8 (iPR8) vaccine provided 100% protection of adult mice against influenza challenge [11]. However, as the 3-week gestation period of mice allowed time for just a single immunization during pregnancy we first tested whether a single immunization with iPR8 alone or with a delta inulin adjuvant (iPR8+Advax) could be used to protect virgin adult mice. Female mice were immunized once intramuscularly (i.m.) with iPR8 100ng alone or with Advax and bled 4 weeks later for anti-PR8 antibodies. IgG1, IgG2a and IgG2b anti-PR8 antibody levels were significantly higher in the iPR8+Advax versus iPR8 alone group (Fig. 1A). This correlated with reduced weight loss (Fig. 1B), lower clinical disease scores (Fig. 1C), and significantly improved survival for mice immunized with iPR8+Advax (survival 7/10, 70%, $p=0.03$) versus iPR8 alone (survival 1/10, 10%) or sham immunization (survival 0/10, 0%) (Fig. 1D). This established that just a single iPR8 dose

when combined with Advax adjuvant was able to protect adult mice making immunization feasible during the short murine gestation period.

Delta inulin adjuvant enhances immunogenicity of vaccine administered during pregnancy

Pregnancy has been shown to be state of relative immunosuppression, necessary to maintain immune tolerance to her fetus [21]. To test the ability of Advax adjuvant to overcome immune suppressive effects of pregnancy, BALB/c mice at 7 days gestation (G7) were immunized with 5µg iPR8+Advax or iPR8 alone then bled 4 weeks post-immunization for measurement of anti-influenza antibodies. The addition of Advax adjuvant to iPR8 antigen resulted in higher titers of anti-PR8 IgM, IgG, IgG1, IgG2a, and IgG3 compared to immunization with iPR8 alone (Fig. 2A). When challenged post-partum, all dams immunized with either iPR8 alone or with Advax were protected and survived whereas the same challenge dose was 100% lethal in control saline-immunized dams (data not shown).

Maternal immunization with Advax-adjuvanted influenza vaccine enhances neonatal protection

The major focus of this study was to test the effect of adjuvanting a maternal influenza vaccine on passive protection of the immunized mother's pups. Maternal antibody acquired by pups through transplacental transfer or breast milk provides a critical mechanism of protection against infection during the period of neonatal immune immaturity [22, 23]. We wished, therefore, to see whether the addition of Advax adjuvant to vaccine administered to pregnant mothers could enhance protection of their unimmunized pups. Pups of G7-immunized dams receiving 5µg iPR8 alone or iPR8+Advax were challenged at 4 weeks of age. No protection (survival 0/13, 0%) was seen in control pups of unimmunized dams (Fig. 2D). Similarly, high mortality and clinical illness was seen in pups of dams immunized with iPR8 alone (survival 5/15, 33%). By contrast, pups of dams that had received iPR8+Advax exhibited complete protection (survival 17/17, 100%, $p<0.0001$) and even continued to put on weight throughout the challenge period (Fig. 2B). Day 14 post-challenge, surviving pups of iPR8-immunized dams had still not regained their pre-challenge weights, whereas pups of iPR8+Advax-immunized dams had increased an average of ~40% from their pre-challenge weight (Fig. 2C).

Serum anti-PR8 IgG titers in pups correlate with serum and breast milk IgG titers in their immunized mothers

To better understand the mechanism whereby immunization of pregnant dams with iPR8+Advax enhanced protection of their unimmunized pups, the pattern of anti-influenza IgG subtypes was studied in the sera and breast milk of dams that had received iPR8+Advax versus iPR8 alone and also in the sera of their pups at day 10 post-birth. Serum anti-PR8 IgG was significantly higher in dams immunized with iPR8+Advax versus iPR8 alone (Fig. 3A). A similar pattern of increased anti-PR8 IgG was observed in the breast milk of dams immunized with iPR8+Advax versus iPR8 alone (Fig. 3B). Finally, the same highly significant pattern of elevation of anti-PR8 IgG of all subtypes, i.e. IgG1, IgG2a, IgG2b and IgG3, was observed in the sera of pups of dams immunized with iPR8+Advax (Fig. 3C).

Delta inulin adjuvant enhances passive protection of pups through enhanced breast milk IgG transfer

It has previously been demonstrated that acquisition of influenza-specific antibody in neonatal mice is predominantly mediated by breast-feeding [23]. We hypothesized that enhanced protection of pups of dams receiving iPR8+Advax could likewise be due to enhanced breast milk transfer of maternal IgG. Cross-fostering studies were performed to identify the mechanism involved. Offspring of iPR8+Advax-immunized dams were transferred at birth to suckle on foster mothers who had received sham-immunization. Conversely, offspring of sham-immunized mothers were transferred at birth to suckle on iPR8+Advax immunized dams. At the time of weaning at 3 weeks of age, maternally acquired anti-PR8 IgG was measured in pup sera. High levels of anti-PR8 IgG1 and IgG2a were seen in pups of sham-immunized dams suckled from birth by iPR8+Advax-immunized dams (Fig. 4A). By contrast, pups of iPR8+Advax-immunized dams suckled from birth with sham-immunized dams showed minimal serum anti-PR8 IgG (Fig. 4A), consistent with breast milk being the major delivery route of anti-PR8 IgG. When pups were challenged at 4 weeks of age, significantly higher levels of survival (survival 15/23, 65%) and reduced levels of clinical disease was seen in the pups of sham-immunized mothers suckled from birth by iPR8+Advax-immunized dams, whereas no survival was seen for pups of iPR8+Advax-immunized dams suckled from birth by sham-immunized dams (0/25, 0%, $p<0001$) (Fig 4B–D). We next asked whether the neonatal Fc receptor (FcRn) [24] was elevated in the gut of pups of iPR8+Advax-immunized dams, thereby allowing these pups to extract a higher percentage of IgG from milk during breast-feeding. However, we found no significant difference in FcRn gene expression as measured by real-time PCR in the proximal duodenum of 10 day old pups of dams immunized with iPR8+Advax versus pups of dams immunized with iPR8 alone (data not shown).

Safety of Advax-adjuvanted influenza vaccine in pregnancy

To assess safety and potential reproductive toxicity of delta inulin adjuvant administered during pregnancy, litter sizes, neonatal weights and teratogenic defects were compared between iPR8 alone and iPR8+Advax immunized dams. No injection site lesions were observed in the Advax-immunized mothers (data not shown). There was no significant difference in litter sizes of between sham immunized and vaccine groups ; saline – mean 5, SD 0.9; iPR8 – mean 4.3, SD 1.5; iPR8+Advax – mean 5.0 SD 1.6 (Fig. 5A). Similarly, there were no significant differences in neonatal weights between sham immunized and vaccine groups; saline – mean 9.0gm, SD 0.6; iPR8 – mean 8.5gm, SD 0.9; iPR8+Advax – mean 8.6gm SD 1.0 (Fig. 5B). No teratogenic defects were observed in any vaccine group. Pups of iPR8+Advax-immunized dams showed normal development, feeding behavior and weight gain through to adulthood (data not shown) consistent with delta inulin adjuvant being free of reproductive or developmental toxicity.

DISCUSSION

The results show that co-administration of a delta inulin adjuvant helps overcome pregnancy-associated immune suppression, resulting in enhanced anti-influenza antibody titers in the immunized dams. This is consistent with our previous findings of enhanced

influenza protection in non-pregnant animals receiving an Advax-adjuvanted influenza vaccine [11]. Higher serum anti-PR8 titers in iPR8+Advax-immunized dams was associated with increased immunoglobulin titers in the breast milk of these dams when compared to those immunized with iPR8 alone. This in turn translated into higher serum IgG titers in the suckling infants of dams immunized with iPR8+Advax. This enhanced maternal transfer of passive immunity via IgG transfer to infants of iPR8+Advax-immunized dams provided enhanced protection when pups of iPR8+Advax-immunized mothers were challenged with a lethal dose of influenza virus one week post-weaning. The critical role of breast-feeding in the enhanced neonatal protection was confirmed in cross-fostering studies where infants of non-immunized mothers were protected providing they were suckled from birth by iPR8+Advax-immunized foster mothers. Protection was lost if infants of iPR8+Advax-immunized mothers were suckled from birth by non-immunized foster mothers, consistent with transfer of IgG by breast milk rather than transplacental transfer playing the dominant role in passive influenza protection in mice, as previously reported [8]. These results confirm that inclusion of a delta inulin adjuvant with influenza immunization administered during pregnancy has beneficial effects on anti-influenza antibody titers for both the mother and her suckling infants translating into enhanced protection of the suckling infant in particular.

Notably, there was minimal protection of pups of mothers immunized with iPR8 alone, even although the iPR8-immunized mothers were themselves protected against a lethal challenge. Surprisingly, this was despite the non-surviving pups of iPR8 alone-immunized mothers having similar pre-challenge serum anti-PR8 IgG titers to their mothers. This may be explained by the fact that immunized mothers, in addition to protective IgG, have the benefit of vaccine-induced IgM and IgA plus anti-influenza memory B and T cells, all of which may contribute to influenza protection in the mother [25]. By contrast their pups are entirely reliant on passively acquired maternal IgG for protection and are thereby likely to require higher thresholds of IgG for protection than seen for their mothers. This is consistent with a study during the 1979 H1N1 epidemic where infants of H1N1-immune mothers, despite having serum H1N1 titers correlating with their mother's titers, had no significant difference in influenza incidence to infants of non-immune mothers, although influenza symptoms were delayed and of shorter duration [22]. Another study similarly reported maternal immunization with an unadjuvanted vaccine did not reduce risk of neonatal influenza-like illness, otitis media visits or outpatient visits [26]. Other studies have suggested that infants of immunized mothers are ~50% less likely to have influenza hospitalizations or illnesses than infants of unimmunized mothers [27–29]. However, it is possible that the observed protective effect on the infants was due to the vaccine reducing maternal viral shedding thereby reducing the risk of an infected mother infecting her infant, rather than the infants' protection necessarily being mediated by vaccine-induced passive immunity.

Our data strongly suggests that a more potent maternal influenza vaccine is needed for protection of babies by passive immunity than is required for protection of the mother herself. Hence it is notable that immunization of pregnant dams with iPR8+Advax provided complete protection for their pups even when challenged a week post-weaning. A delta inulin-adjuvanted influenza vaccine for pregnant mothers might thereby help reduce the period of vulnerability between when an infant loses passively acquired maternal immunity

and when they are able to respond normally to influenza immunization. In human infants this vulnerability extends up to 6 months of age when they start to respond to influenza immunization [30, 31], consistent with infants being most likely to die from influenza or be hospitalized between birth and 6 months of age [32–34]. Opportunities to close this neonatal window of vulnerability include, as shown here, development of more potent vaccines for use in pregnant mothers, combined with more effective influenza vaccines able to overcome neonatal immune immaturity that could thereby be used in newborns [35], rather than having to wait till infants are 6 months of age. Interestingly, a previous study in neonatal mice suggested that maternal immunization with a high-dose inactivated H3N2 influenza vaccine was able to protect their 2 week old unimmunized pups not only against the homologous virus, but also to a lesser extent against heterologous H1N1 and H2N2 but not B group viruses, protection the authors attributed to maternally acquired cross-neutralizing antibodies acquired through the breast milk [8]. Whilst we only showed enhanced protection against the homologous virus in the current studies, future studies will examine whether the Advax adjuvant is similarly able to enhance passive protection of unimmunized neonates against heterologous influenza viruses. This should be a promising area of study as Advax adjuvant has already been demonstrated to induce broad cross-protective humoral responses against flavivirus antigens with an Advax-adjuvanted inactivated Japanese encephalitis vaccine providing robust protection against a West Nile virus challenge [14].

A critical consideration when developing influenza vaccines for use in pregnant women are safety, both for the mother and her child. Prior to the last decade, influenza immunization guidelines only recommended immunization for women with high risk of respiratory complications, to be given during the second or third trimester (reviewed in [36]). Better appreciation of the increased morbidity for pregnant women in low risk categories contracting influenza [37, 38], in 2004 led to extension of U.S. recommendations to all pregnant women irrespective of risk category [39]. During the 2009 H1N1 pandemic there was an over-representation of pregnant women among severe and fatal cases [40]. Despite expanded recommendations, vaccine uptake by pregnant women remains low at 10–35% in a U.S. study [41] and <2% in a Hong Kong study [42]. Low uptake reflects poor knowledge among pregnant women of the current recommendations and the excellent safety in pregnancy of current vaccines [42, 43]. Clinical trial data suggests no increased risk of pregnancy complications or adverse fetal outcomes in women immunized in the second or third trimester [44–46] with, if anything, lower risks of premature birth for immunized subjects [47]. Despite more limited data on adjuvanted vaccine safety in pregnancy, widespread use of squalene-adjuvanted vaccines in Europe during the 2009 H1N1 pandemic resulted in significant exposures of pregnant women, with no reports of safety issues [47, 48]. This is an important consideration as in event of a pandemic due to high pathogenicity avian influenza, adjuvants will almost certainly be critical to creation of an effective vaccine [49] thereby necessitating their use in pregnant women. While delta inulin adjuvant has been tested and shown safe in human influenza vaccine trials [19], it has yet to be given to pregnant women. However, inulin in its soluble form has been safely administered intravenously in doses of tens of grams to human patients as part of a routine kidney function test, including to pregnant women and newborn babies with no adverse effects reported [10]. The current study data further supports the safety of inulin in pregnancy when

it is in the Advax adjuvant delta inulin particulate form. Notably, no teratogenic or other adverse effects on litter size, fetal weights or development were observed after administration of Advax adjuvant to pregnant dams.

Whilst the mechanism of action of delta inulin adjuvant is not yet fully characterized, progress has been made in this direction [12, 14, 50]. The working hypothesis is that delta inulin acts through regulation of antigen presenting cells leading to enhanced co-stimulation of antigen-specific T and B cells in the absence of the excess inflammation associated with other adjuvants. This would help to explain delta inulin's remarkable lack of reactogenicity [19]. This, combined with its high adjuvant potency, supports Advax's development for use in influenza vaccines targeting vulnerable populations, including pregnant women and young children.

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HIGHLIGHTS FOR REVIEW

- A single immunization with influenza vaccine plus delta inulin adjuvant at gestational day 7 provided enhanced passive protection of neonates
- Enhanced protection provided by delta inulin adjuvant demonstrated to be due to enhanced transfer of immune IgG in breast milk resulting in higher anti-influenza IgG levels in suckling pups.
- First demonstration that delta inulin adjuvant is safe and well tolerated when administered to pregnant dams.
- Study highlights the potential to use a delta inulin adjuvanted influenza vaccine during pregnancy to enhance maternal immunogenicity and also enhance passive protection of infants.

Figure 1. Advax adjuvant enhances immunogenicity and protection of a single influenza vaccine dose in virgin female mice

Female BALB/c mice ($n = 10/\text{group}$) were immunized i.m. with iPR8 100ng alone (white bars) or with Advax (black bars). Blood samples were collected 4 weeks post-immunization and mice then challenged with 1×10^4 TCID₅₀ ($8 \times \text{LD}_{50}$) of PR8 virus. Shown are; pre-challenge anti-PR8 antibodies measured by ELISA (mean $\pm$ SEM) (A), post-challenge bodyweight changes (mean $\pm$ SEM) (B), post-challenge sickness score (mean $\pm$ SEM) (C), post-challenge survival rate (D), with number of survivors/number of challenged mice

shown in parenthesis. +, no survivors; ns, not significant; *, $p < 0.05$; ***, $p < 0.001$. Data representative of 3 independent experiments.

Figure 2. Effect on protection of pups of immunization of pregnant dams

Pregnant BALB/c mice were injected on G7 with iPR8 5 μ g alone (white bars) or with Advax adjuvant (black bars). Serum anti-PR8 titers in postpartum mothers (A). Pups were challenged with 5000 TCID₅₀ PR8 virus at 4 weeks of age and shown are bodyweight changes of survivors (mean $\pm$ SEM) (B), mean bodyweight of animals at day 14 post-challenge (mean + SEM) (C), and survival rate (D), with number of survivors/number of challenged mice shown in parenthesis. **, $p < 0.01$. Data representative of 3 independent experiments.

A Maternal Sera

B Breast Milk

C Pup Sera

Figure 3. Enhanced passive immunity in offspring of dams receiving Advax-adjuvanted vaccine Pregnant mice on G7 were injected with iPR8 5 μ g (white bars) or iPR8+Advax (black bars). Ten days postpartum sera and breast milk of dams and sera of infants were collected and anti-PR8 antibody levels determined (mean + SEM) (**, $p < 0.01$; ***, $p < 0.001$). Data representative of 3 independent experiments.

Figure 4. Effect of cross-fostering on acquisition of passive immunity by pups
 Pregnant BALB/c mice ($n = 3/\text{group}$) were immunized on G7 with saline or iPR8+Advax. On the day of birth, offspring born to immune and naïve mothers were cross-fostered. At 4 weeks of age, pups were bled and then challenged with 5000 TCID₅₀ PR8 virus. Shown are pre-challenge anti-PR8 IgG1 and IgG2a antibodies (mean + SEM) for dams (white bars) and pups (black bars) (A), bodyweight changes (B), and sickness scores (C), of challenged pups and survival post-challenge (D); number of survivors/number of challenged mice shown in parenthesis. +, no survivors.

Figure 5. Delta inulin adjuvant does not exhibit reproductive or developmental toxicity
 Pregnant dams immunized on G7 with either iPR8 antigen alone or iPR8+Advax had no significant differences in mean litter size (A), or body weight of their pups at 4 weeks of age (B), when compared to pups of sham immunized dams.