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Maternal Transfer of RSV Immunity in Cotton Rats Vaccinated During Pregnancy

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Abstract

Respiratory Syncytial Virus (RSV) is the leading cause of pneumonia and bronchiolitis in infants, resulting in significant morbidity and mortality worldwide. There is currently no RSV vaccine. Although maternal serum antibodies against RSV are efficiently transferred through placenta protecting human infants from RSV-induced disease, this protection is short-lived and the methods for extending and augmenting protection are not known. The objective of this study was to develop an animal model of maternal RSV vaccination using the *Sigmodon hispidus* cotton rat. Naïve or RSV-primed female cotton rats were inoculated with live RSV and set in breeding pairs. Antibody transfer to the litters was quantified and the offspring were challenged with RSV at different ages for analysis of protection against viral replication and lung inflammation. There was a strong correlation between RSV-neutralizing antibody (NA) titers in cotton rat mothers and their pups, which also correlated with protection of litters against virus challenge. Passive protection was short-lived and strongly reduced in animals at 4 weeks after birth. Protection of litters was significantly enhanced by inoculating mothers parenterally with live RSV and inversely correlated with the expression of lung cytokines and pathology. Importantly, vaccination and boosting of naïve mothers with the live RSV produced the highest levels of NAs. We conclude that maternal vaccination against RSV in the cotton rat can be used to define vaccine preparations that could improve preexistent immunity and induce subsequent transfer of efficient immunity to infants.

Keywords

RSV; cotton rat; maternal immunization; pregnancy

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1. Introduction

Respiratory Syncytial Virus (RSV) is the leading cause of pneumonia and bronchiolitis in infants, resulting in significant morbidity and mortality worldwide [1]. There is no vaccine approved for RSV infection. A vaccine for future mothers that can extend passive protection of babies in the critical period between 2 and 6 months after birth and beyond would be highly desirable [2]. In addition, such a vaccine could have a secondary protective effect in the household by preventing spread of RSV within families [3]. In an era when maternal vaccination is increasingly considered to be a valuable strategy to protect both the mother and infant against RSV, further research is needed to evaluate the potential beneficial effect of different vaccine formulations. However, characterization of maternal vaccines could be complicated since transfer of immunity from mother to infant is dependent on a large range of complex variables that need to be defined in a preclinical model of RSV vaccination.

Antibodies against RSV are found universally in adult sera [4], and pooled adult sera have been shown to be protective in high-risk infants against RSV infection and disease [5]. In addition, studies have shown evidence that the higher the levels of maternal anti-RSV neutralizing antibody (NA) titers, the longer the protection of the babies. It was reported that the severity of RSV disease in infants <9 months old was reduced when levels of maternal antibodies were higher [6] and that higher levels of antibodies at the time of birth correlated with later times of infant infection [7].

Maternal immunization has shown efficacy for influenza, tetanus, and pertussis [2, 8] and this vaccination regimen is particularly convenient and effective in resource-limited settings. In babies, there is a selective transfer of antibody isotypes from mothers, with the IgG1 subtype predominating [9]. In addition, maternal vaccination has been shown to be safe for both mother and infants. For example, vaccination of pregnant women against influenza during all trimesters is now widely used in the U.S.A. and has not been associated with increased risk of preterm or small gestational age births [10].

The cotton rat model is extensively used for testing RSV vaccines and therapeutics. The strength of this model for studying passive immunity has been well recognized as evidenced by the development of the antibody-based prophylactic therapies (*i.e.*, Respigam® and Synagis®) against RSV [11, 12]. Previous studies demonstrated that immune cotton rats transfer maternal immunity through placenta and by nursing [13]. In this work, we characterize the parameters and conditions that will be important for optimizing vaccines that will enhance maternal transfer of immunity to RSV. Study of different vaccination strategies could identify one that selectively enhances natural mechanisms of the transfer of maternal immunity and extend the period of protection. This work establishes the basis for a model that attempts to reflect maternal immunization and passive transfer of immunity in humans with the expectation that it will permit us ultimately to enhance maternal immunity and to develop protection against RSV in the older infants and children.

2. Materials and methods

2.1. Animals

Inbred *Sigmodon hispidus* cotton rats were obtained from a colony maintained at Sigmovir Biosystems, Inc. (Rockville, MD). Three to five-week-old female animals were used for vaccination experiments. Animals were pre-bled before being included in the study to rule out the possibility of preexistent antibodies against RSV. Animals were housed in large polycarbonate cages and fed a standard diet of rodent chow and water *ad libitum*. The colony was monitored for antibodies to paramyxoviruses and rodent viruses, and no such antibodies were found. All studies were conducted under applicable laws and guidelines and after approval from the Sigmovir Biosystems, Inc. Institutional Animal Care and Use Committee.

2.2. Viruses and viral assays

The prototype Long strain of RSV was obtained from American Type Culture Collection (ATCC VR-26, Manassas, VA). Virus was propagated in HEp-2 cells and serially plaque-purified to reduce defective-interfering particles. The single pool of virus containing $10^{7.6}$ pfu/ml was used for all experiments. To adjust the dose, stock was diluted with PBS for intranasal (i.n.) and intramuscular (i.m.) immunization. Viral titers in the lungs and in the nose of RSV-infected infant cotton rats were determined as described elsewhere [14] and adjusted by the weight of the lung portion or expressed per nose.

2.3. RSV neutralizing antibody (NA) assay

RSV NA titers were measured by 60% plaque reduction assay using four-fold dilutions of heat-inactivated serum samples against the RSV/A/Long strain (25–50 PFU) in Hep-2 cells incubated in 24-well plates at 37° C after overlaying the wells with 0.75% methylcellulose medium. After 4 days of incubation, the overlay was removed and the cells were fixed in 2.5% glutaraldehyde solution containing 0.1% crystal violet for one hour, rinsed, and air-dried. The corresponding reciprocal NA titers were determined as previously indicated [15]. The limit of detection (LOD) of this assay was 4.32 Log₂ or a 1:20 dilution of the serum [14].

2.4 Cytokine expression by Real-time PCR

Total RNA was extracted from homogenized lung tissue using the RNeasy purification kit (QIAGEN). One µg of total RNA was used to prepare cDNA in a volume of 20µl (QuantiTect Reverse Transcription Kit from Qiagen). cDNA was diluted to 0.1µg /ml and 3 µl were used for each 25µl real-time PCR reaction (QuantiFast SYBR Green PCR Kit from Qiagen) with final primer concentrations of 0.5 µM. Reactions were set up in 96-well plates and amplifications were performed on a Bio-Rad iCycler (MyiQ Single Color). Delta *Ct* method was used to calculate relative gene expressions, that were normalized to β-actin as a housekeeping gene [16].

2.5. Lung Histopathology

Lungs were dissected and inflated with 10% neutral buffered formalin to their normal volume, and then immersed in the same fixative solution. Following fixation, lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). An average total score was determined for each group based in four parameters of pulmonary inflammation: peribronchiolitis (inflammatory cell infiltration around the bronchioles), perivascularitis (inflammatory cell infiltration around the small blood vessels), interstitial pneumonia (inflammatory cell infiltration and thickening of alveolar walls), and alveolitis (cells within the alveolar spaces). Slides were scored blindly on a 0–4 severity scale as previously described [16].

2.6. Experimental design

During the first experiment (outlined in Figure 1A), two groups of female cotton rats (3–5 weeks of age; 10 rats/group) were “primed” by intranasal (i.n.) or intramuscular (i.m.) inoculation with live RSV (10^5 PFU/animal) (Figure 1). A third group of 5 females remained untreated as controls (unprimed). Two weeks post-priming, all females were mated in separate cages with naïve, age-matched males. Before delivery, all females were bled for the determination of total anti-RSV NA. Each litter was allocated randomly to one of 4 different subgroups, corresponding to pups challenged at 1, 2, 3, and 4 weeks after birth. Each subgroup had 13 to 22 cotton rat pups from at least two different mothers. To prevent pups from being rejected by the mother and to keep 3–4 week age groups at the same nursing level, all pups reaching age 21 days were weaned. Each subgroup of pups was challenged under isoflurane anesthesia with RSV/A/Long (10^5 pfu/pup) using a corrected volume for intranasal inoculation: 1- and 2-week pups with 25 μ l, 3-week pups with 40 μ l, and 4-week pups with 50 μ l. Pups were bled and sacrificed at day 4 p.i. The left lobe of the lung and the noses were homogenized in 1 ml of HBSS +10%SPG +1% Fungizone + 0.1% Gentamicin for the determination of the nose and lung viral titers as previously described [14].

In a second experiment (outlined in Figure 2A), 25 female cotton rats, 3-weeks old, were separated into 5 groups. Animals in Group A remained naïve throughout experiment and gave birth to pups that were subsequently challenged with RSV. Animals in groups B, C, and D were “primed” by i.n. infection with RSV/A/Long (10^5 pfu/100 μ l/rat) on day 0. Two weeks later, animals in groups C and D were vaccinated i.m. with live RSV/A/Long (10^5 pfu). Animals in group E were not primed, but were vaccinated i.m. with live RSV/A/Long (10^5 pfu) at the same time as groups C and D. At week 5, all cotton rats in each group were separated into single cages and paired with a naïve male cotton rat. On week 7, pregnant females in groups D and E, were boosted i.m. with the same live RSV preparation. Serum samples from all females were obtained after the first vaccination (week 6), after the boost but before delivery (week 8), and again, before the second delivery (week 20). The litters from each group were subdivided into two subgroups that were challenged with RSV/A/Long (10^5 pfu/animal) at one or four weeks of age and sacrificed 4 days after infection. After delivery of the first litter, all females were maintained in our facility without further manipulation. A second programmed pregnancy of the original females in the described

groups was conducted 16 weeks “post-priming” and the subsequent litters were challenged with RSV in a manner identical to the one described above.

3. RESULTS

3.1. Priming of cotton rat mothers with live RSV protects infant cotton rats against RSV infection

Female, naïve cotton rats were separated into three groups, *i.e.*, uninfected (unprimed) or inoculated with live RSV via the i.n or i.m. route (Figure 1A, long arrow). Two weeks later, all females were paired with a naïve male. Serum from all female rats were collected at 4 weeks after priming and showed that NA titers were equivalent ($\sim 10 \text{ Log}_2$) (Supplemental Figure 1). All females delivered between 4–8 pups per litter.

Pups from naïve and RSV-primed mothers were challenged with RSV at different times after birth (1, 2, 3, or 4 weeks) and sacrificed 4 days after infection, when the peak of RSV viral replication in cotton rats occurs [14]. Pups, born by RSV-primed mothers (either i.n. or i.m.), were protected against lung RSV replication (Figure 1B). However, an inverse correlation between the age of the pup and the level of protection was noted. Pups challenged with RSV during the first week of life showed almost complete protection from viral replication in the lung (Figure 1B, 1wk). Pulmonary protection, although still detectable in pups challenged at 4 weeks of age (~ 10 -fold reduction in virus titer compared to control, unprimed mothers), was dramatically decreased compared to pups challenged at 1 week of age. As expected, maternally-transferred protection was weaker in the nose, than in lungs of the litter. Nasal RSV titers showed a reduction during the first week of life, although this reduction did not reach significance (Figure 1C).

Rapid decline in RSV NAs was also evidenced by the analysis of the serum of the litters. During the first week of life, all pups showed high levels of NA (Figure 1D) that correlated with the high level of protection seen in the lung and the significant protection seen in the nose (Figure 1B and 1C). However, circulating NA decreased thereafter and stabilized at a titer of ~ 64 within a month. NA titers declined faster in animals born to mother primed by i.n. RSV infection compared to pups born to mothers primed by i.m. RSV inoculation. Serum neutralizing antibodies also decreased faster in females primed i.n. (Supplemental Figure 1). A comparison between serum NA titers in one-week old pups and serum NA titers of their mothers just prior to delivery revealed that females vaccinated i.m. are more efficient for transferring NA to the pups than females vaccinated via the i.n. route ($r=0.8$ (open circles) *vs.* $r=0.2$ (closed circles) (Figure 1E).

3.2. RSV Vaccination of seropositive mothers boosted their serum NA

Most adults have circulating anti-RSV antibodies present in their sera [4]. Thus, a more realistic preclinical animal model for testing the efficacy of maternal vaccination is one that uses females previously exposed to RSV. Thus, we first prepared “primed” groups of female cotton rats infected with the RSV/A/Long strain i.n. (animals in groups B–D, Figure 2A) at week 0. Vaccination of primed Groups C and D was performed by i.m. injection of live RSV two weeks after priming. Control animals included primed and unvaccinated animals (Group

B) and unprimed vaccinated animals (Group E), as well as completely naïve animals (Group A). Animals in Groups D and E were boosted i.m. during their pregnancy (5 weeks after the first vaccination), with the same live RSV/A/Long preparation.

NA titers of mothers were analyzed using serum samples collected three weeks after the first vaccination (6th week), near the time of delivery (8th week), and on week 20 (~12 weeks after the first delivery or before the second delivery, see below) (Figure 2B). Females primed by i.n. RSV infection (Group B) and not vaccinated thereafter showed levels of RSV NA of ~256 (8 Log₂) on week 6, and this titer remained relatively constant in serum samples obtained on the 8th and on the 20th weeks (Figure 2B and Supplemental Figure 1). Vaccination in primed animals (Groups C and D) significantly improved the NA response, increasing NA titer by the time of the delivery (week 8th) to ~1,024 in animals vaccinated and boosted during pregnancy (Group D). Boosting females during pregnancy also maintained NA titers significantly higher on week 20 than in not boosted females (Group C) ($p<0.0001$). Importantly, naïve mothers that were vaccinated and boosted (Group E) generated the highest levels of NA titers (~4,096) at the time of delivery and maintained high titers even through week 20. Altogether, these data demonstrate that pre-existent immunity achieved in intranasally infected animals can be improved by vaccination of mothers before and during pregnancy. However, a comparison of the NA titers of naïve animals (Group E) vs. primed, vaccinated animals (Groups B, C, and D) revealed evidence for an inhibitory mechanism that prevents optimal induction of protective NA in previously infected mothers.

3.3. Immunity afforded by vaccination of primed cotton rats strengthens the protection of the pups

To determine whether the vaccination of previously primed cotton rats improved antibody transfer to their litters, we measured NA in sera from pups born to mothers in the previously defined groups when pups were one (Figure 2C) or four (Figure 2D) weeks old. Consistent with the levels of NA in the mothers, pups from naïve mothers that were vaccinated and boosted (Group E) exhibited the highest NA titer (~4,096) one week after birth (Figure 2C). The NA titer in Group E decreased, but still remained the highest among all the groups when measured four weeks after birth (NA titer of ~512; Figure 2D). Importantly, the group of primed mothers that was vaccinated and boosted during pregnancy (Group D) transferred significantly higher levels of NA than groups of primed mothers that were primed and vaccinated only once (Group C) or animals that were primed but not vaccinated (Group B). NA titers of animals born to mothers that were only primed (Group B) decreased sharply to non-protective levels during the four weeks of life (compare Figure 2C and 2D). This indicates that among the primed animals, those that were born to mothers vaccinated prior to and boosted during pregnancy maintained the highest levels of NA titers at four weeks of life.

One week-old pups and 4 week-old animals born to mothers from each group were challenged with RSV to determine whether protection correlated with the measured NA titers that were passively transferred from the mothers to the litters. Nose and lung viral titers were measured on day 4 post-infection (Figure 3A and B, Lung and Nose). A strong inverse correlation between the passively transferred NA titers and RSV titers was

evidenced. Thus, the highest protection was observed in the pups born to unprimed, but vaccinated and boosted mothers (Group E). In this group, complete protection of the lung and nose tissues was evidenced in one week-old pups, and almost complete protection of the lung and strong reduction of the nose viral titers in 4 week-old animals. Importantly, vaccination and boosting during pregnancy of primed females (Group D) increased and extended protection of the litters in this group when compared to the primed and vaccinated group (Group C) or to mothers that were only primed (Group B, compare lung and nose titers in these groups in Figure 3A and B). Together, these results indicate that maternal vaccination with boosting during pregnancy enhances maternal transfer of NA, and increases protection of the litters.

3.4. Cytokine gene expression analysis

RSV virus titers in the lung correlated with the expression of IL-6 mRNA that was highest in one week-old pups born to naïve to RSV mothers (Group A), while IL-6 mRNA levels were reduced in RSV-infected pups in all other primed and/or vaccinated groups (Figure 4A, IL-6). In animals challenged at 4 week of age, reduced IL-6 expression was only seen in those groups derived from mothers not primed, vaccinated, and boosted (Figure 4B, IL-6, Group E). IFN- γ and IL-4 mRNA gene expression are host genes tested to evaluate Th1 and Th2 profile of gene expression, respectively, in the lung after challenge (Figure 4A and B) [17]. As for IL-6, IFN- γ gene expression was strongly reduced in one week-old animals born to primed only (group B), primed and vaccinated (groups C), primed, vaccinated and boosted (group D), or unprimed vaccinated and boosted rats (Group E), when compared with pups born to naïve mothers (Group A). Interestingly, IFN- γ mRNA expression was lower in naïve, 4 week old juveniles, when compared to its expression in naïve, one week-old pups (compare IFN- γ expression for Group A in Figure 4A and B). This difference could be the result of developmental immunological changes that occur in the lung of cotton rats. A significant reduction in the expression of IFN- γ mRNA was seen in 4-week-old juveniles born to vaccinated and boosted mothers that were not primed (Group E). The expression of IL-4 mRNA in the lungs of all experimental age groups was low and comparable to levels found in animals without any maternally-derived protection (Figure 4A and B, IL-4), and indicates that animals born from primed or vaccinated mothers are not predisposed to develop a Th2 profile upon subsequent RSV challenge.

3.5. Lung Histopathology

Lung histopathology was scored on day four post-challenge of one-week-old pups or 4 week old juveniles from each of the vaccine groups (Figure 5A). There was a small reduction in the lung pathology recorded in one-week-old pups that received maternal immunity (Groups B–E) compared to those born from naïve mothers (Group A, Figure 5A, $p < 0.05$). In four-week-old pups, a small reduction in pathology was detected only in the juveniles born from unprimed, vaccinated, and boosted mothers (Group E, see Figure 5A and 5B, compare panel a and b vs c and d), whereas no significant improvement in pathology in the juveniles born from the other vaccinated groups was noted. Importantly, no evidence of enhanced pathology was observed in any of the pups born from immune mothers (Groups B–E). Thus, none of the vaccination protocols resulted in a predisposition of the pups or juveniles to enhanced disease.

3.6. Transfer of RSV immunity to a second litter

Because RSV immunity in humans is short-lived [18], we set out to explore the possibility that immunity in cotton rats also wanes with time, resulting in decreased protection of pups born to mothers that first encountered infection more than three months earlier. A consistent reduction in protective immunity transferred from mothers to litters was evidenced in all groups of one-week pups and 4-weeks juveniles (Figure 6B), delivered as a consequence of a second pregnancy (Figure 6A and B, respectively). This reduction in protective immunity correlated in most of the vaccinated groups with a reduction in NA titers in the litters (Figure 6A and B, NA panels). Lung protection in animals born from mothers primed, vaccinated, and boosted during the first pregnancy was significantly higher than that found in animals born from primed but unvaccinated females. These data indicate that maternal vaccination can extend protection even beyond the first pregnancy.

4. Discussion

The data presented demonstrate that cotton rat is a useful model to dissect the outcome of maternal vaccination against RSV. We have demonstrated that RSV infection of female cotton rats induces immunity by production of RSV NA that is efficiently transferred to their litter. An early study of influenza immunization of pregnant women showed that active transport of IgG produced umbilical-cord antibody levels that were higher than those in maternal serum [19]. And although similar data for RSV maternal immunization in humans is not available, recent results in mother-babies pairs show that maternal transfer of RSV NA is also highly efficient [20].

Our study shows that similar results can be inferred in cotton rats. Furthermore, we demonstrate that the passive immunity transferred was sufficient to afford high levels of upper and lower respiratory tract protection during the first week of life. However, as in humans, there is a rapid decay of protection that correlated strongly with the loss of circulating maternal RSV NA in the infants. The half-life of maternal antibodies in humans vary across populations, ranging from 20 to 80 days [21–23], whereas in cotton rats was determined to be 7 days [13]. Aside from these differences, we have established in detail the kinetics of NA decay in cotton rats and its strong impact and correlation with protection of the litter. We have determined that 4 weeks after birth is the relevant time to test the efficiency of maternal vaccination in this model since passive protection transferred from primed mothers is strongly reduced (*i.e.*, lungs) or nonexistent (*i.e.*, nose).

This vaccination study performed in primed, pregnant cotton rats evidenced the inhibition of vaccine efficacy in animals with pre-existent immunity. Experiments conducted here (Figure 1) compared females primed by either i.n. infection or i.m. inoculation with live virus. While both types of priming induced measurable maternally-transferred immunity, the data presented indicate that the mechanisms of immunity induced by i.m. priming with live RSV *vs.* intranasal RSV infection are different. A more rapid decline in maternally-transferred NA titers was seen in pups born to i.n.-primed mothers compared to pups born to mothers primed via i.m. RSV-inoculation (Figure 1D), and this also is evidenced in the mothers (Supplemental Figure 1). Moreover, an increase in NA titers in pups following RSV boosting of the mother was very strong only in animals born to mothers primed i.m., and not

i.n. (Figure 3 and data not shown). These differences may point to an important vaccine blocking mechanism elicited by intranasal RSV infection, an effect that is overcome to some degree, by repeated intramuscular vaccinations (Figure 2, Group D). While having little relevance to the real-life situation, where all humans are primed via natural intranasal RSV infection, this experimental regimen represents an interesting opportunity to dissect immune modulatory mechanisms of RSV infection.

Previous vaccination studies performed in humans using a subunit vaccine showed ~4-fold increase in serum NA titer [24–26], whereas only a 1.4-fold increase was seen with the same vaccine in pregnant women [21]. In our study, we induced a 4-fold increase in pre-existent immunity using our i.m. live RSV inoculation (from 8 Log₂ to 10 Log₂), which extended immunity in the litter (Figure 3B) when compared to that provided by mothers that were primed only. A recent study on RSV transplacental antibody transfer and kinetics in mother-infant pairs showed that some of these pairs had RSV NA titers between 12 and 14 Log₂ [20]. Although such high titers are rare in humans, the goal of an effective RSV vaccination program targeted to the adult population. Using the cotton rat as a model, we have demonstrated that only those animals inoculated and boosted i.m. *but not primed* (Figure 2, Group D), are able to achieve high NA titers (Figure 2C and D), conferring the strongest upper and lower respiratory tract protection to viral replication (Figure 3A and B) and lung pathology (Figure 5) to their litters during the fourth week of life. Thus, we predict that a vaccine that could increase preexistent immunity provided by natural i.n. infection (NA of ~250 or 8 log₂) ~8-fold compared to that seen in vaccinated and boosted naïve cotton rats (NA of 4000 or 12 Log₂) would likely be efficacious in a clinical setting. We have chosen a dose of RSV of 10⁵ pfu i.n. for priming animal since this is the routine dose used for challenge in vaccination studies in cotton rats and, in addition, it is known to induce life long protection of the lung [14, 16, 17]. Intramuscular injection with live virus was used as our testing vaccine since it was demonstrated to be safe and efficacious in naïve animals, but unable to overcome the inhibition imposed by maternal antibodies [27]. Although live attenuated RSV vaccines would not be used for pregnant women with pre-existing immunity, the current study presents a good model system to indicate a potential advantage to use live vector platform for parenteral vaccination.

The search for efficacious and safe RSV vaccine has been compounded by many difficulties, including a high-risk target population with immature immunity, the short-lived immunity generated by natural RSV infection, and the potential of a vaccine to elicit dangerous immunological conditions such as “vaccine enhanced disease”. We and others previously demonstrated that the cotton rat model replicates the human vaccine enhanced disease in response to vaccination with the RSV Lot 100 vaccine [17, 28]. Lot 100-vaccinated cotton rats also induced very high levels of Th1 and Th2 cytokines in the lungs after challenge of Lot 100-immunized animals with RSV [17]. These cytokine levels were higher than those in the lungs of naïve cotton rats challenged with RSV. Thus, analysis of cytokine gene expression serves as a useful surrogate marker of safety. We tested whether a maternal vaccination protocol that efficiently reduces viral replication in lung could predispose the litter to enhanced pathology upon viral challenge. We found that all animals born from prime, and prime and vaccinated mothers experienced a reduction of the inflammatory cytokines IL-6 and IFN- γ (Th1) during the first week of life upon RSV challenge. In

addition, there was no evidence of stronger expression of IL-4 mRNA in lungs of challenged animals, indicating that maternal vaccination as presented, does not predispose the litter to a Th2-type of immune response. Although this reduction in the expression of IFN- γ could imply a delay for mounting a strong adaptive RSV immune response in young infants, the strong passive immunity should protect them from the first RSV season when they are most vulnerable. Moreover, it seems that both IL-6 and IFN- γ expression in the lungs of litters from primed, or primed and vaccinated mothers challenged at 4 weeks of life, are enhanced when compared to litters challenged at one week of age. These differences could possibly suggest immunological or physiological developmental changes in the lung that modify the response to RSV infection.

Lung histological analysis showed reduced pathology in those litters with the strongest RSV passive immunity (Group E) when compared to litters from naïve mothers. However, the model showed that only marginal benefit to lung pathology is achieved in litters from primed, or primed and vaccinated mothers, which could suggest that higher levels of passive NA antibodies are needed to reach this threshold of protection.

The cotton rat has become an important model for RSV vaccine and therapy testing due to its high susceptibility to infection by this virus [14]. In this model, different human cohort scenarios of RSV infection have been modeled, including RSV disease in infants, elderly, and immunosuppressed individuals [29]. The model has accurately predicted efficacy, safety, and dose of antibody immunoprophylaxis, as well as lack of efficacy of antibody therapy [11]. Finally, the cotton rat recapitulates the pathology associated with the FI-RSV vaccine-enhanced disease [28]. We have now shown in the cotton rat model that a protocol of immunization of primed mothers using vaccination before the pregnancy and boosting during the pregnancy, brings significant advantage to the litter with respect to protection from RSV challenge. However, we also show that vaccination without the boosting (Group B), did not establish any clear protective benefit for the litter. Our data suggest that intramuscular vaccination and subsequent i.m. boosting during the pregnancy period renders the most benefit. In addition, we show that the protection achieved by vaccination remains effective, albeit with a small decay, over time and also imparts benefit to the 2nd litter.

Overall, we have presented evidence that vaccination during pregnancy could be efficiently used to enhance maternal immunity and prevent RSV infection extending protection. Importantly, we also established the conditions to determine whether a vaccine could improve pre-existent immunity in the adult population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Maternal transfer of immunity in cotton rats. (A) Scheme of the protocol of RSV priming and transfer of immunity by RSV-primed cotton rat mothers. Female animals were primed with live RSV A/Long (10^5 pfu/100 μ l/animal), given intranasally (i.n.) or intramuscularly (i.m.), or left unprimed. Females were set in mating pairs and four weeks later they gave birth to litters that were subsequently analyzed by RSV challenge at 1, 2, 3, and 4 weeks after birth. Number of pups in each group was between 12–22 animals. (B) Lung RSV titers obtained 4 days after challenge of litters from mothers unprimed, primed intramuscularly or

intranasally at the indicated weeks after birth. Bars represent mean $\pm$ SE. Significance was assessed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. # $p<0.0001$; * $p<0.01$; •, $p<0.05$. (C) Nose viral titers on day 4 post-challenge. (D) Decay of maternal RSV NA titers during the life of infant cotton rats whose mothers were unprimed or primed with RSV intranasally or intramuscularly. (E) Correlation between RSV NA titers in sera of mothers before delivery and in sera of their litters on day 11 after birth. ● represents pups from mothers primed i.n. Correlation coefficient was $R=0.23$ for 59 pups from 10 litters. ○ represents pups from mothers primed i.m. Correlation coefficient was $R=0.83$ for 98 pups from 14 litters.

Figure 2.

RSV neutralizing antibodies analysis in mothers and litters after vaccination during pregnancy. (A) Protocol of priming and maternal vaccination against RSV. Female cotton rats in groups A remained naïve. Female cotton rats in groups B to D were infected with RSV i.n. (Prime) whereas animals in group E were left unprimed. Two weeks after priming, animals in Groups C–E were inoculated with live RSV i.m. (10^5 pfu, Vaccine). All females were set in mating pairs 5 weeks post-infection and those in Groups D and E were boosted with the same dose of live RSV i.m. during pregnancy (week 7th, Boost). Females started deliveries on week 9 after priming. (B) Measurement of RSV NA in serum samples of females from the different groups collected on week 0 (before priming), on week 6 (after vaccination), on week 8 (after boosting and before delivery), and on week 20 (11 weeks after delivery). NA titers were reported as Log₂ of the reciprocal dilution of serum samples that achieved 60% reduction of the total plaques. N=5–7 females per group. (C) NA titers in 1 week-old pups born from group of mothers described in A. Number in parenthesis represents total amount of animals per group. (D) NA titers in 4 weeks-old juveniles. Comparison of responses between pups in group B and pups in groups C, D and E was assessed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. # $p<0.0001$; * $p<0.01$; •, $p<0.05$.

Figure 3.

Protection against RSV in newborn and juvenile cotton rats born from vaccinated mothers. Quantification of lung and nose viral titers in samples of RSV-challenged cotton rats born from the groups of mothers (defined in Figure 2A) and sacrificed on day 4 post challenge. (A) One week-old pups; (B) Four week-old juveniles. Bar represent the mean $\pm$ SE. Significance of vaccination during pregnancy was evaluated between Group B (primed only group) and groups D or E (primed, vaccinated, boosted; or not primed, vaccinated, and boosted, respectively); N=13–25 pups per group. Comparison of responses between pups in

group B and pups in groups C, D and E was assessed by one-way ANOVA followed by Student-Newman-Keuls post hoc test. # $p<0.0001$; + $p<0.005$; •, $p<0.05$.

Figure 4.

Analysis of cytokine gene expression in the lung after RSV challenge of 1-week-old pup (A) and four-week-old juvenile (B) cotton rats born from mothers in the previously described experimental groups (Figure 2A). Expression of IL-6 and IFN- γ (Th1) and IL-4 (Th2) were tested. Each bar represents the mean using at least 2 pups from each litter with total of 5–8 animals per group $\pm$ SE. Significance differences of vaccination effects during pregnancy was evaluated between group B (primed only group) and groups D or E (primed, vaccinated, boosted; or not primed, vaccinated and boosted, respectively) by one-way ANOVA followed by Student-Newman-Keuls post hoc test. *, $p < 0.01$; •, $p < 0.05$.

Figure 5.

Lung histopathology. (A) Lung pathology scores (Average Total Score/animal) recorded for one week-old and four week-old cotton rats in the indicated vaccination groups (Figure 2A) after challenge. Lung samples were collected on day 4 post-challenge with RSV/A/Long. naive represents a group of animals of the respective age that were not challenged and were used as normal control. Significant reduction in the total score was recorded when compared Group A with groups B to E as indicated. *, $p < 0.01$; •, $p < 0.05$. (B) Representative lung histology from four-week-old juvenile in group E (a and b) and group A (c and d).

Figure 6.

Comparison of RSV protection and NA titers between first (grey bars) and second litter (black bars) in cotton rats born from mothers vaccinated as in Figure 2A. (A) One week-old pups; (B) Four week-old juveniles. Viral titers in the lung and nose cotton rats measured on day 4 post-infection. Significant differences between the first and second litter were determined by Student *t*-test. #, $p < 0.0001$; +, $p < 0.005$; *, $p < 0.01$; •, $p < 0.05$. NA (right graphs) represents the titers of neutralizing antibodies in the indicated groups. #, $p < 0.0001$; ▼, $p < 0.001$; +, $p < 0.005$; *, $p < 0.01$; •, $p < 0.05$.