A virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A provides protection against viral challenge without priming for enhanced disease in cotton rats

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Background Non-replicating respiratory syncytial virus (RSV) vaccine candidates could potentially prime for enhanced respiratory disease (ERD) due to a T-cell-mediated immunopathology, following RSV infection. Vaccines with built-in immune response modifiers, such as Toll-like receptor (TLR) ligands, may avoid such aberrant imprinting of the immune system.

Methods We developed reconstituted RSV envelopes (virosomes) with incorporated TLR4 ligand, monophosphoryl lipid A (RSV-MPLA virosomes). Immune responses and lung pathology after vaccination and challenge were investigated in ERD-prone cotton rats and compared with responses induced by live virus and formaldehyde-inactivated vaccine (FI-RSV), a known cause of ERD upon RSV challenge.

Results Vaccination with RSV-MPLA virosomes induced higher levels of virus-neutralizing antibodies than FI-RSV or live virus infection and provided protection against infection. FI-RSV, but not RSV-MPLA virosomes, primed for increases in expression of Th2 cytokines IL-4, IL-5, IL-13, and Th1 cytokine IL-1b, 6 hour–5 days after infection. By contrast, RSV-MPLA virosomes induced IFN-γ transcripts to similar levels as induced by live virus. Animals vaccinated with FI-RSV, but not RSV-MPLA virosomes showed alveolitis, with prominent neutrophil influx and peribronchiolar and perivascular infiltrates.

Conclusion These results show that RSV-MPLA virosomes represent a safe and immunogenic vaccine candidate that warrants evaluation in a clinical setting.

Keywords Adjuvant, cotton rat, enhanced respiratory disease, monophosphoryl lipid A, respiratory syncytial virus, vaccine, virosomes.

Introduction

Respiratory syncytial virus (RSV) has been recognized as an important vaccine target for over 60 years; however, there is no vaccine on the market today. A clinical trial conducted in the 1960s evaluated a formalin-inactivated, alum-absorbed RSV preparation (FI-RSV) as a possible vaccine candidate.[1] Infants vaccinated with FI-RSV who subsequently acquired a natural infection, developed enhanced respiratory disease (ERD), especially in the youngest group of vaccinees who were naïve to RSV prior to vaccination, which led to the death of two of these children.[2,3] In those two infants, severe bronchiolitis and alveolitis were observed with an influx of neutrophils and mononuclear cells into the lungs.[2] Although the vaccine significantly increased RSV-specific antibody titers in 95–100% of vaccinees, the induced antibodies failed to neutralize the virus in half of the patients.[4,5] Studies in mice demonstrated that impaired Toll-Like receptor (TLR) signaling by the vaccine results in low antibody affinity maturation and, consequently, in the formation of poorly virus-neutralizing antibodies.[6] Furthermore, it has been shown that FI-RSV induces a predominantly Th2-skewed immune response.[7,8] Therefore, the objective in the development of non-replicating RSV vaccines is to generate a formulation that induces high titers of neutralizing antibodies without priming for T-cell responses that induce lung pathology upon natural infection.

One way to induce protective immunity without priming for immunopathology involves the addition of a Th1-skewing adjuvant to the vaccine. The detoxified

lipopolysaccharide (LPS) derivative monophosphoryl lipid A (MPLA) has been shown to skew the immune response to a Th1-phenotype through the activation of TLR4.[9] Additionally, MPLA has an acceptable safety profile when co-administered with vaccine antigens in multiple clinical trials and is the only TLR ligand currently being used as an adjuvant in licensed human vaccines.[10–12] Furthermore, it has been shown that addition of MPLA to FI-RSV alleviates the symptoms of RSV-enhanced disease.[13]

In a recent study in mice, we showed that reconstituted RSV viral envelopes (virosomes) containing viral membrane proteins and incorporated MPLA but lacking the nucleocapsid represent a promising vaccine candidate.[14] Immunization with these RSV-MPLA virosomes led to induction of high levels of neutralizing antibodies and a balanced Th1/Th2 phenotype compared with FI-RSV immunization. Mice immunized with RSV-MPLA virosomes cleared the virus after infection and showed no signs of immunopathology.

Even though mice are a valuable model to assess vaccine-induced immune responses, they are not permissive for RSV replication and display a different form of ERD compared with humans. Infiltration of eosinophils is a hallmark of FI-RSV-induced ERD in mice, while in the clinical trial conducted in the 1960s, analyses of autopsy samples indicated excessive infiltrating neutrophils and only scarce influx of eosinophils.[8,15] Cotton rats (*Sigmodon hispidus*), although still being semi-permissive, are more permissive for RSV than mice, and this animal model also displays FI-RSV-induced lung immunopathology with neutrophil infiltration.[15,16]

The cotton rat was used for pre-clinical evaluation of the prophylactic antibody palivizumab and has become the small-animal model of choice for RSV vaccine development.[17] Recently, key cotton rat cytokine genes were sequenced enabling the analysis of Th1/Th2 cytokine profiles using qPCR.[18] It was shown that immunization of cotton rats with FI-RSV not only induces increased Th2 cytokine expression, but also stimulates expression of several Th1-associated cytokines after live virus challenge.[13] The combination of the permissiveness of the cotton rat for RSV, the occurrence of ERD and the new opportunity to profile Th1/Th2 cytokine responses make this animal model very suitable to study the safety and efficacy of RSV-MPLA virosomes.

Here, we show that RSV-MPLA virosomes induce a superior immune response compared with FI-RSV or non-adjuvanted RSV virosomes. It induces increased virus-neutralizing antibody levels compared with levels induced by FI-RSV or non-adjuvanted RSV virosomes, a strongly reduced Th2 response compared with responses induced by FI-RSV without inducing alveolitis with influx of neutrophils in the lungs after challenge. These results, combined with the responses to immunization we observed in mice, show that RSV-MPLA virosomes represent a safe and immunogenic RSV vaccine candidate that warrants further evaluation in a clinical setting.

Materials and methods

Ethical statement
Animal experiments were approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, according to the Dutch Animal Protection Act (permit number DEC 5239D). Immunizations and challenges were conducted under isoflurane anesthesia, and every effort was made to minimize suffering of the animals.

Cells and virus
Respiratory syncytial virus strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, the Netherlands). The virus was grown in HEp-2 cells (ATCC, CL-23, Wesel, Germany) in roller bottles in HEp-2 medium: DMEM (Invitrogen, Breda, the Netherlands) supplemented with Pen/Strep, L-glutamine, sodium bicarbonate, HEPES, sodium pyruvate, 1X non-essential amino acids (all from Invitrogen) and 2% FBS (Lonza-Biowhittaker, Basel, Switzerland) and purified on sucrose gradients as described before.[14]

Vaccine formulations
Respiratory syncytial virus virosomes were generated as described previously and contain the viral proteins F and G, and to some extent M protein.[19] Briefly, the RSV membrane was dissolved in 100 mM 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC) in 5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7·4 (HNE), and the nucleocapsid was removed by ultracentrifugation. The supernatant was applied to a dried film of a 2:1 mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE; Avanti Polar Lipids, Alabaster, AL, USA) at a ratio of 850 nmol lipid per mg of supernatant protein. For incorporation of MPLA, MPLA from *Salmonella minnesota* Re 595 (Invivogen, Toulouse, France) dissolved in 100 mM DCPC in HNE was added to the protein lipid mixture at 1 mg MPLA per mg supernatant protein, incubated for 15 minute at 4°C, filtered through a 0·1-μm filter, and dialyzed in a sterile Slide-A-lyzer (10 kD cut-off; Thermo Scientific, Geel, Belgium) against HNE buffer pH 7·4. After dialysis, virosomes were kept at 4°C. A detailed procedure for production and characterization of the RSV-MPLA virosomes has been described before.[14]

FI-RSV was produced as reported before.[15] FI-RSV was diluted with HNE to contain 5 μg of RSV protein in a 50 μl volume.

Animals and immunizations
Female outbred cotton rats (Hsd:Cotton Rat) of 4–6 weeks old were obtained from Harlan (Indianapolis, IN, USA).

Cotton sats received 50 µl RSV virosomes or RSV-MPLA virosomes intramuscularly containing 5 µg of protein. Control cotton rats received 100 µl (10^6 $TCID_{50}$) intranasally, 50 µl of HNE intramuscularly, or 50 µl (5 µg viral protein) of FI-RSV intramuscularly. Vaccinations were given on day 0 and 21. On day 49, cotton rats were challenged with 10^6 $TCID_{50}$ RSV intranasally. At the time of immunization and challenge, blood was drawn by retro-orbital puncture. Six hours or 5 days after challenge, cotton rats were sacrificed and blood was drawn by heart puncture. Lungs were removed aseptically, and one of the primary bronchi was ligated just below the tracheal bifurcation with suture wire. Approximately 20 mg of this lobe was removed and stored in 1 ml of RNA later (Qiagen, Venlo, the Netherlands) at −20°C for RNA isolation. The remainder of this lobe was kept on ice in HEp2 medium containing 2% FBS, for virus titration. The other half of the lung was fixed in 4% formaldehyde in PBS under 20 cm of water pressure to preserve the structure of the lungs for lung histopathology analyses. Control cotton rats used for RNA expression analyses were sacrificed as described above without prior challenge.

IgG antibody ELISA

Respiratory syncytial virus-specific serum IgG titers were determined using standard protocols[19] using horseradish peroxidase–coupled goat anti-mouse IgG (1030-05; Southern Biotech, Birmingham, AL, USA) which was cross-reactive for cotton rat IgG.

Virus titration and microneutralization assay

Virus titers were determined by $TCID_{50}$ as described previously.[14] RSV virus neutralization titers were determined by incubation of twofold serially diluted heat-inactivated (30 minute, 56°C) cotton rat serum with 70 $TCID_{50}$ of RSV for 2 hour and subsequent titration of this mixture on HEp-2 cells as described before.[14] The neutralization titer was

calculated with the Reed & Muench method,[20] as the dilution that neutralizes infection in 50% of the wells.

Lung histopathology

The inflated lungs were embedded in paraffin and 4-µm slices were cut. The slides were then stained with hematoxylin and eosin (H&E) using standard procedures. Subsequently, perivasculitis, peribronchiolitis, and alveolitis were assessed by light microscopy. Lungs from three animals per group were evaluated, and the histology was judged in a blinded fashion by two different investigators. Neutrophils were identified based on morphology. Numbers of neutrophils were determined in four areas per slide and three animals per group and counted by two different investigators. The average of these counts was considered to represent an objective quantification of neutrophil influx.

Lung RNA isolation

Lung samples stored in RNA later (Qiagen) were transferred to tubes containing 300 µl RLT buffer (Qiagen) and homogenized using disposable pestles with a pestle motor (VWR, Amsterdam, the Netherlands). Subsequently RNA was isolated with an RNeasy mini kit (Qiagen) according to the manufacturer's protocol. After isolation, RNA concentration was determined on a Nanodrop (Thermo scientific, Wilmington, DE, USA).

cDNA synthesis and qPCR

cDNA synthesis and qPCR were performed using the Verso cDNA synthesis kit (Acros Organics, Geel, Belgium) and Absolute QPCR SYBR Green Mix (Westburg, Leusden, the Netherlands) according to the manufacturer's protocols. Cotton rat cytokine sequences were obtained from NCBI[18] and used to generate primers that yield products of approximately 125 bp (Table 1). PCR analyses were run on an Applied Biosystems AB7500 Real-Time PCR system (Applied Biosystems, Nieuwerkerk ad IJssel, the Netherlands)

Table 1. Primers for cotton rat cytokine RT-PCR

|	Sense primer	Antisense primer	Product length
Th1			
IL-1b	GTGCTGTCTGACCCATGTGAGC	GCCACTGGTATCTTGGTGCTGC	122
IFN-γ	CATCAACACCATCAGGGCGGAC	TGACAGCTTTGCGCTGGACC	125
IL-12-p40	CCACTCACGTCTGCTGCTTCAC	ACCGGCCAGAGTAATTTGCTGC	125
Th2			
IL-4	GCTTCCCAGGTGCTTCGCAAG	TGGAGACAGACTGCTGATGCCC	120
IL-5	GGGGCACTGTGGAAACGCTATTC	GCAGGTAATCCAGGAACTGCCG	117
IL-9	TGTGACCAGTTGCTTGTGTCTTCC	ACCCGATTGAAAAGAACCGAGAACC	126
IL-13	TGCCTCGATGCATGTCTCCTCC	GCTGTCAGGTCCACGCTCCATAC	124
Housekeeping gene			
β-actin	CCCCAAGGCCAACCGTGAAAAG	CACAATGCCAGTGGTACGACCAG	124
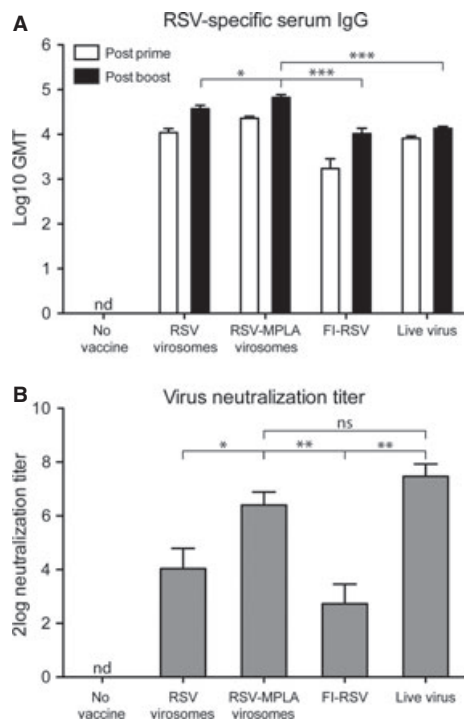
Figure 1. Antibody responses to respiratory syncytial virus (RSV) vaccination. Cotton rats were immunized twice (day 1, prime and 21, boost) with virosomes, FI-RSV or live virus. (A) On day 21 and 49, blood was drawn and RSV-specific IgG titers in serum were determined. (B) Virus neutralization titer of serum on day 49 (Mann–Whitney *U*-test *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 2. *In vivo* virus clearance after challenge. Cotton rats were challenged with live RSV at day 49. (A) Five cotton rats per group were sacrificed at 6 hours post-challenge and lung virus titers were determined by $TCID_{50}$. (B) Seven cotton rats per group were sacrificed at 5 days post-challenge and lung virus titers were determined by $TCID_{50}$. Values below 10 $TCID_{50}$ were not detectable (Mann–Whitney *U*-test **$P < 0.01$, ***$P < 0.001$). RSV, respiratory syncytial virus.

with the following conditions: 15 minute 95°C, 40 times 30 second 95°C, 30 second 60°C followed by a melt curve. Relative transcript abundance was calculated using the 2^{-ddC_t} method comparing C_t (cycle threshold) values from the immunized and challenged cotton rats to non-immunized, non-challenged cotton rats corrected for C_t values for the housekeeping gene β-actin.

Statistical analyses

All statistical analyses were performed with GRAPHPAD PRISM 5.00 for Mac OSX, (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Statistical significance was assessed using a Mann–Whitney *U*-test. *P*-values of 0·05 or lower were considered to represent significant differences.

Results

Immunogenicity of RSV-MPLA virosomes

Virosomes were produced and analyzed as described before.[19] To determine the immunogenicity of RSV virosomes and RSV-MPLA virosomes, serum samples from immunized cotton rats, collected on day 21 and 49, were analyzed for RSV-specific IgG antibodies (Figure 1A). Immunization with RSV virosomes induced a significantly
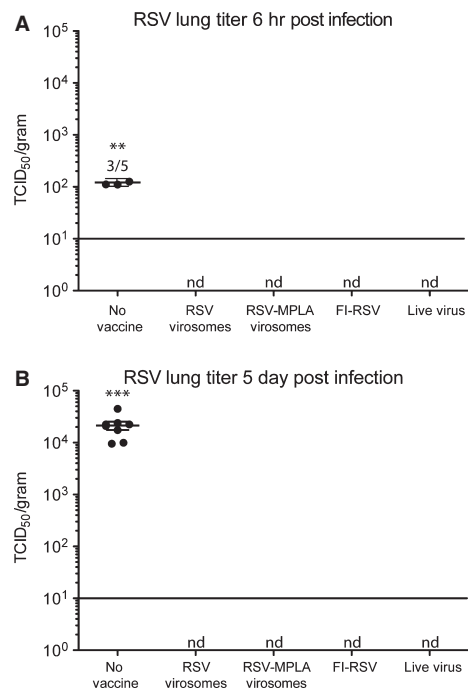
higher RSV-specific IgG titer in serum than immunization with FI-RSV or live virus. The inclusion of MPLA in the RSV virosomes significantly increased the antibody titer to an approximately twofold higher level.

To determine the virus-neutralizing capacity of the RSV-specific serum antibodies, a microneutralization test was performed (Figure 1B). Although FI-RSV induced similar levels of RSV-specific antibodies compared with immunization with live virus, the antibodies induced by FI-RSV had a significantly lower capacity to neutralize the virus (Figure 1B). Sera from cotton rats immunized with RSV virosomes did not show increased capacity to neutralize the virus compared with sera from FI-RSV-immunized animals. Sera from cotton rats immunized with RSV-MPLA virosomes demonstrated significantly higher levels of neutralizing antibodies compared with sera from animals immunized with FI-RSV or non-adjuvanted virosomes.

Protection against RSV infection

To determine whether immunization with MPLA-adjuvanted RSV virosomes could also induce a protective immune response *in vivo*, animals were immunized and challenged with live RSV and lung virus titers were determined (Figure 2). At 6 hour post-infection, RSV was recovered
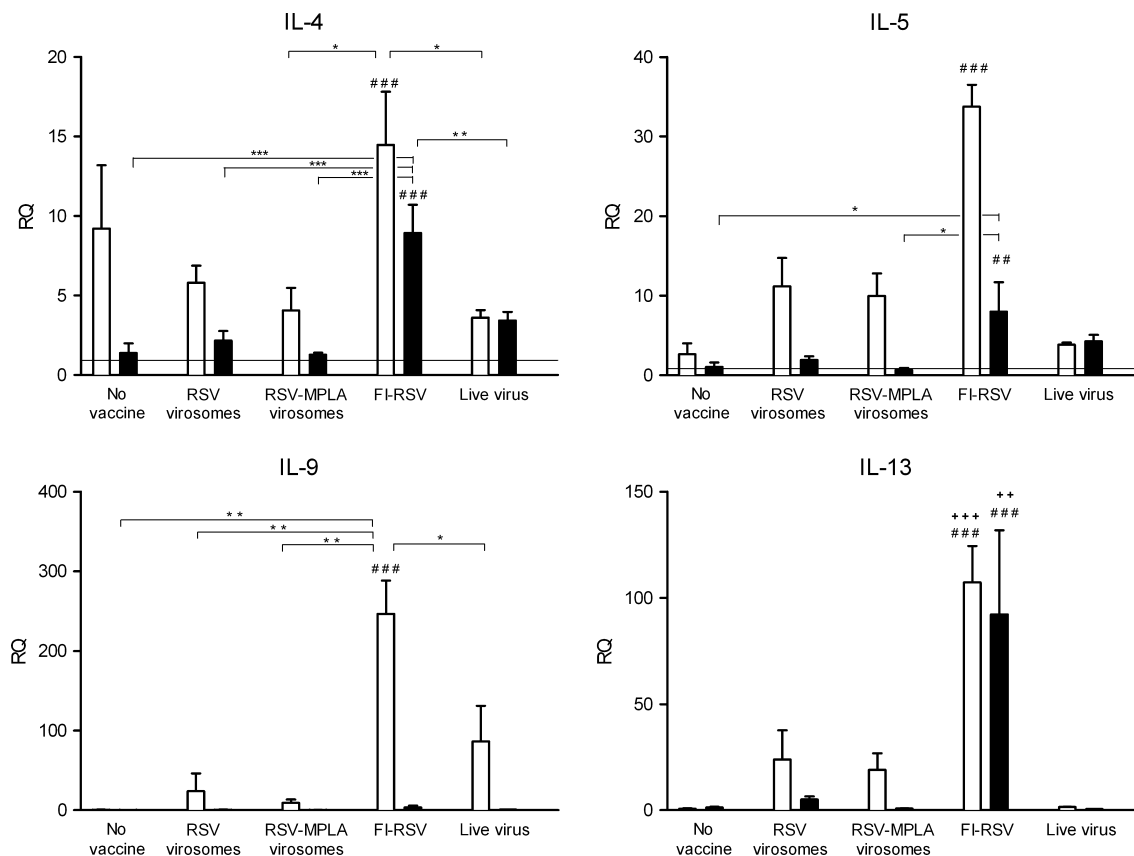
Figure 3. Th2-type cytokine expression in the lungs of vaccinated and challenged animals. Cotton rats were vaccinated and challenged as in Figure 2, and at 6 hours (open bars) and 5 days (solid bars) post-infection, lung cytokine expression was determined by qPCR. Values are relative quantities (RQ) compared with naïve cotton rats (horizontal line at RQ = 1; Mann–Whitney U-test *$P < 0.5$, **$P < 0.01$, ***$P < 0.001$ comparing vaccine group to vaccine group. #$P < 0.5$, ###$P < 0.001$ comparing expression values of the vaccinated and challenged cotton rat to expression in naïve cotton rats).

from three of five control animals with titers of approximately 10^2 TCID$_{50}$/gram lung. None of the immunized animals had detectable virus in their lungs at this time point (Figure 2A). At 5 days post-challenge, increased RSV viral titers were detected in the lungs of all control animals, to above 10^4 TCID$_{50}$/gram lung, indicating virus replication in these animals. In contrast, no RSV was detected in the lungs of the animals immunized with RSV virosomes, RSV-MPLA virosomes, FI-RSV, or live virus (Figure 2B).

Local immune responses upon RSV infection

To analyze whether RSV-MPLA virosomes prime for immune responses associated with ERD, cotton rats were vaccinated and challenged as described above and cytokine gene expression levels in the lungs of the animals were assessed by qPCR (Figures 3 and 4).

At 6 hour after infection, cotton rats immunized with FI-RSV showed a significant increase in Th2-associated cytokines IL-4, IL-5, IL-9, and IL-13 mRNA expression (Figure 3). At this time point, cotton rats immunized with

RSV virosomes showed increased levels of IL-5 mRNA as well. In contrast, animals immunized with RSV-MPLA virosomes or live virus did not show significant increases in the expression of Th2-associated cytokines at this early time point. At 5 days post-infection, IL-4, IL-5, and IL-13 expression remained high in animals immunized with FI-RSV. In contrast, animals immunized with RSV virosomes, RSV-MPLA virosomes, or live virus only showed background mRNA levels of these cytokines.

Animals immunized with FI-RSV also showed a significant increase in the Th1-associated cytokine IL-1b, but not IL-12 or IFN-γ mRNA (Figure 4). Animals immunized with live virus showed early increases in expression levels of IL-1b, IL-12, and IFN-γ mRNA, while non-immunized animals had increased expression levels of IL-1b and IL-12, but not IFN-γ mRNA at 6 hour post-infection. The levels of IL-12 and IFN-γ mRNA were significantly higher in animals immunized with live virus than in animals immunized with RSV virosomes, but similar to levels in animals immunized with RSV-MPLA virosomes. At 5 days post-infection, all the
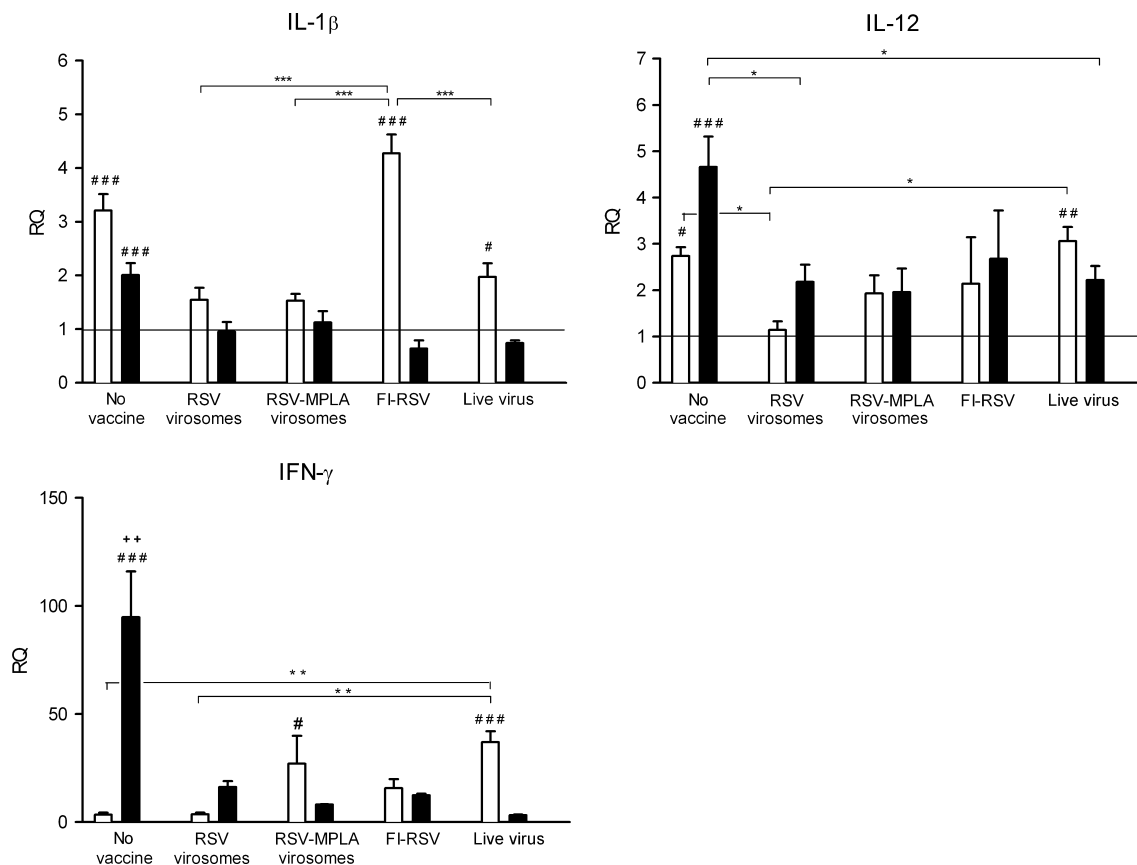
Figure 4. Th1-type cytokine expression in the lungs of vaccinated and challenged animals. As Figure 3 but for Th1-type cytokines. Open bars represent relative transcription abundance at 6 hours post-challenge and solid bars at 5 days post-challenge.

Th1-type cytokine expression levels in the immunized cotton rats had returned to background levels, that is, levels similar to non-immunized, non-infected animals. Non-immunized animals, however, showed increased levels of IL-12 and IFN-γ at 5 days post-infection.

Immunopathology upon RSV infection

To assess whether RSV-MPLA virosomes primed for the induction of lung pathology upon infection, we examined the lungs of immunized and challenged animals by means of histology. The lungs of non-immunized animals showed no signs of alveolitis, and the peribronchial and perivascular regions were also free of infiltrates (Figure 5A,B). In contrast, the lungs of cotton rats immunized with FI-RSV showed prominent alveolitis as well as peribronchial and perivascular infiltrates (Figure 5C,D). The lungs of animals immunized with RSV virosomes also showed some signs of alveolitis and infiltrates in the peribronchial and perivascular regions (Figure 5E,F). In contrast, cotton rats immunized with RSV-MPLA virosomes showed no sign of alveolitis or infiltrates (Figure 5G,H) and were comparable to non-immunized animals or animals that received live virus immunization (Figure 5I,J).

Finally, infiltrating neutrophils in the alveoli, a hallmark of ERD in both cotton rat and humans,[15] were enumerated. The lungs of cotton rats immunized with RSV-MPLA virosomes contained low numbers of infiltrates similar to those seen in non-immunized animals or animals immunized with live virus. Although we observed an increased number of infiltrating neutrophils in the lungs of the cotton rats immunized with RSV virosomes, the numbers were significantly lower than the numbers induced by immunization with FI-RSV (Figure 6).

Discussion

The induction of ERD by FI-RSV in the clinical trial conducted in the 1960s followed by observations that also other, non-adjuvanted, inactivated vaccine formulations can prime for Th2-type responses and ERD upon RSV infection has tempered enthusiasm for further development of these types of vaccines.[21] Recent insights in the importance of TLR signaling in immunomodulation suggest that addition of TLR ligands to vaccines represents a powerful approach to steer immune responses toward the desired phenotype and have revived the development of inactivated RSV vaccines.
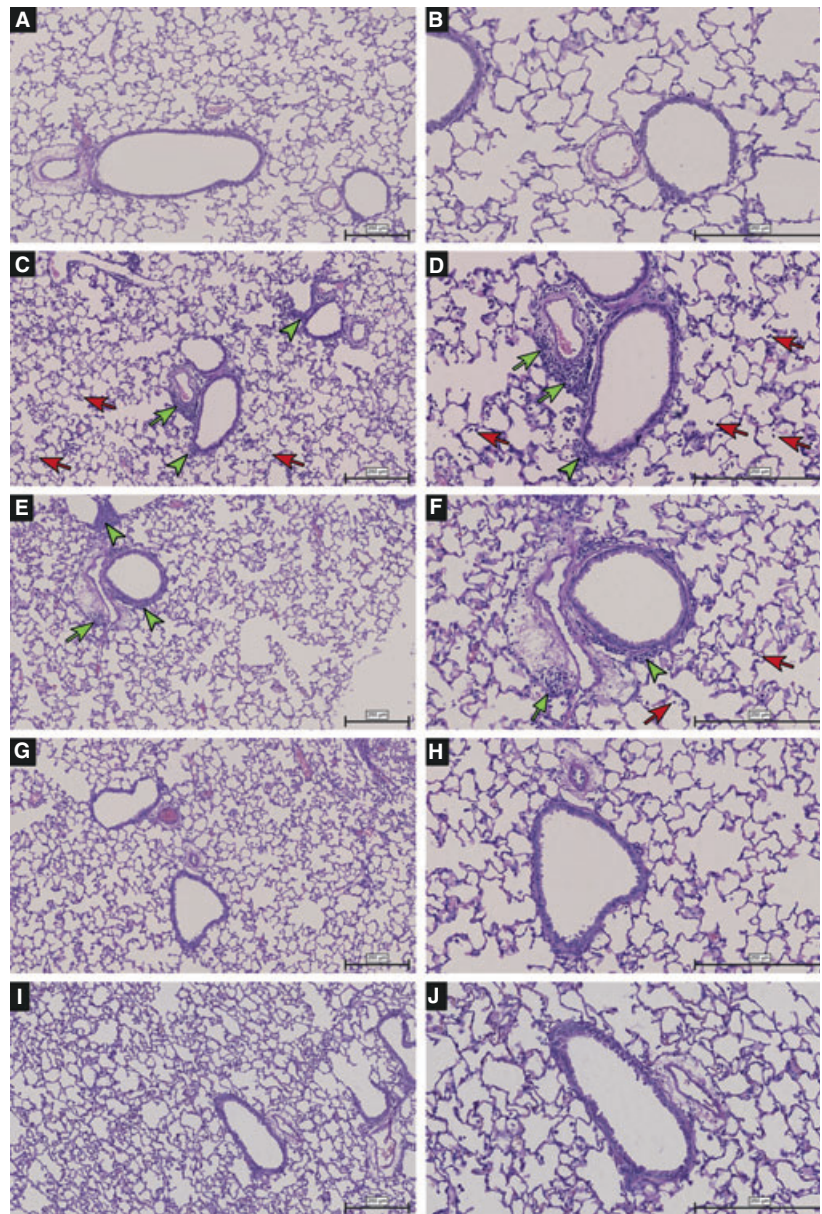
Figure 5. Cotton rat lung pathology. Cotton rats were vaccinated and challenged as in Figure 2. At 5 days post–challenge, lungs were removed, fixed in formalin under 20 cm water pressure to preserve the structure of the lungs, embedded in paraffin and stained with H&E. (A, B) No vaccine. (C, D) FI-RSV. (E, F) RSV virosomes. (G, H) RSV-MPLA virosomes. (I, J) Live virus. Green arrows, perivascular infiltrates. Green arrowheads, peribronchial infiltrates. Red arrows, alveolar infiltrates.

Here, we show that a non-replicating virosomal RSV vaccine, with incorporated MPLA, induces virus-neutralizing immune responses without priming for excess Th2-type responses or ERD in RSV-permissive and ERD-prone cotton rats.

In cotton rats immunized with FI-RSV and subsequently challenged with live virus, we observed a strongly upregulated expression of Th2 cytokines including IL-4, IL-5, IL-9, and IL-13. Studies in mice have shown that expression of these cytokines in lungs is implicated in allergen-induced asthma, rhinitis, and anaphylaxis.[22–25] IL-5, for example, directly activates eosinophils and is therefore an important factor in allergic reactions. IL-9 induces IL-13 expression in the lungs, affecting airway epithelium and contributing to signs of lung pathology.[26] Furthermore, IL-4 and IL-13 upregulate vascular cell adhesion molecule-1 (VCAM-1), which induces migration of eosinophils and lymphocytes. Additionally, upregulation of IL-13 induces mucus secretion in the lungs.[27] Therefore, it is likely that the expression of Th2 cytokines in the lungs of cotton rats, immunized with FI-RSV and subsequently challenged with live virus, may have contributed to the observed alveolar, perivascular, and peribronchial infiltrations.

The exact mechanism by which FI-RSV skews the immune response to a Th2 phenotype is unknown and most likely multifactorial. For example, antigen alteration by formalin[28]
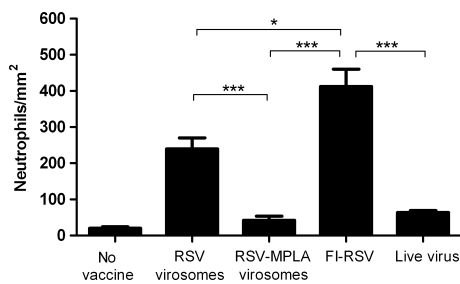
Figure 6. Alveolar infiltration with neutrophils. Lungs were treated as in Figure 5, and the numbers of neutrophils per mm^2 were determined by light microscopy in four areas per slide and three animals per group. (Mann–Whitney *U*-test *P < 0·5, **P < 0·01, ***P < 0·001). MPLA, monophosphoryl lipid A; RSV, respiratory syncytial virus.

and the presence of cell culture proteins[29] and alum[30,31] have all been shown to contribute to Th2-associated cytokine expression and ERD. RSV virosomes, on the other hand, are generated from untreated, purified virus, do not contain alum as an adjuvant, and therefore do not induce excessive Th2 cytokine expression.

Respiratory syncytial virus-monophosphoryl lipid A virosomes stimulated the expression of the Th1-associated cytokine IFN-γ, but not of Il-1b or IL-12. In contrast, priming with live virus resulted in expression of all of these three Th1 cytokines after infection, reflecting a more Th1-skewed response than the response induced by RSV-MPLA virosomes. In a previous study in Th2-prone BALB/c mice, we also observed induction of a balanced Th1/Th2 response by RSV-MPLA virosomes.[14] In the latter study, upon immunization of the animals with RSV-MPLA virosomes, we observed higher levels of virus-neutralizing, Th1-signature, IgG2a antibodies and IFN-γ expression, compared with the corresponding responses in mice immunized with FI-RSV. However, similar to the observations made in the present study, the Th1-skewing effect was more pronounced after priming with live virus than after immunization with RSV-MPLA virosomes. This is in agreement with the findings of Boukhvalova *et al.*,[13] who added MPLA to FI-RSV and, after immunization and subsequent challenge in cotton rats, did not observe a strong Th1-skewed response either. They did, however, note a dampening of the pronounced Th2-type response induced by FI-RSV alone.

Comparison of the results obtained using RSV virosomes versus RSV-MPLA virosomes demonstrates that MPLA is essential for complete prevention of ERD. Even though the expression of Th2 cytokines in cotton rats immunized with non-adjuvanted RSV virosomes was much lower than that in FI-RSV-immunized animals, these animals did display signs of lung pathology. It is possible that these neutrophils were attracted through complement activation, which may have occurred as a result of induction of antibodies with low affinity and low neutralizing capacity (Figure 1), leading to

the deposition of immune complexes in the lungs. This may lead to complement fixation, activation, and chemo-attraction of neutrophils by complement factor fragments such as C3a, C4a, and C5a.[32,33] C3a may additionally induce bronchoconstriction, another symptom of ERD.[2,34] Importantly, immune complex deposition has been demonstrated to play an important role in ERD in mice, and immune complex-dependent C4d deposition has been found in lung biopsies from the children who died of ERD in the trial conducted in the 1960s.[35] Unfortunately, there are, to our knowledge, currently no reagents available to directly investigate immune complex formation and complement fixation in cotton rats.

As indicated before, FI-RSV induces low-affinity antibodies that have poor virus-neutralizing capacity.[4,6] Accordingly, our data indicate that not only FI-RSV, but also non-adjuvanted RSV virosomes, induced antibodies with a lower *in vitro* capacity for neutralization of RSV, compared with antibodies induced by RSV-MPLA virosomes or live virus. Possibly, this points to a lower antibody affinity maturation induced by FI-RSV or non-adjuvanted RSV virosomes. In this respect, TLR4 activation by MPLA in RSV-MPLA virosomes or, for that matter, TLR3 activation by RNA in replicative virus are likely to induce proper antibody affinity maturation yielding antibodies more capable of virus neutralization. In the context of inactivated RSV vaccines, it therefore appears that proper TLR activation is essential and sufficient to ensure the prevention of ERD upon viral challenge.

Apart from stimulating the induction of high levels of high-affinity antibodies, TLR signaling also improves persistence of the antibody response.[36] Previous reports[15] and our data (Figure 2) show that also immunization with FI-RSV gives rise to an antibody response capable of virus neutralization *in vivo*. This is most likely due to the high abundance of these antibodies. Primary immunization with FI-RSV and non-adjuvanted RSV virosomes already induced significant levels of antibodies that were slightly, but significantly, increased by a booster immunization (Figure 1). With respect to the latter, it is possible that administration of the booster immunization at a later time point would have given a more pronounced booster response. However, the lack of TLR signaling upon immunization with FI-RSV for example may cause these antibodies to wane rapidly after the second immunization. In comparison with FI-RSV, RSV-MPLA virosomes, through their ability to activate TLRs, may induce a more sustained high level of antibodies, but this remains to be further investigated.

Taken together, our results show that RSV-MPLA virosomes induce high levels of virus-neutralizing antibodies, which are able to confer protection from RSV infection in cotton rats. Immunization with RSV-MPLA virosomes does not lead to increased expression of Th2-associated cytokines

implicated in the induction of ERD. These data demonstrate the efficacy and safety of RSV-MPLA virosomes as a possible RSV vaccine candidate and warrant further evaluation in clinical trials.

Conflict of interest

Funding

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