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Efficacy and safety of an intranasal virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A in mice and cotton rats

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ABSTRACT

Respiratory syncytial virus infection remains a serious health problem, not only in infants but also in immunocompromised adults and the elderly. An effective and safe vaccine is not available due to several obstacles: non-replicating RSV vaccines may prime for excess Th2-type responses and enhanced respiratory disease (ERD) upon natural RSV infection of vaccine recipients. We previously found that inclusion of the Toll-like receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) in reconstituted RSV membranes (virosomes) potentiates vaccine-induced immunity and skews immune responses toward a Th1-phenotype, without priming for ERD. As mucosal immunization is an attractive approach for induction of RSV-specific systemic and mucosal antibody responses and TLR ligands could potentiate such responses, we explored the efficacy and safety of RSV-MPLA virosomes administered intranasally (IN) to mice and cotton rats. In mice, we found that incorporation of MPLA in IN-administered RSV virosomes increased both systemic IgG and local secretory-IgA (S-IgA) antibody levels and resulted in significantly reduced lung viral titers upon live virus challenge. Also, RSV MPLA virosomes induced more Th1–skewed responses compared to responses induced by FI-RSV. Antibody responses and Th1/Th2-cytokine responses induced by RSV-MPLA virosomes were comparable to those induced by live RSV infection. By comparison, formalin-inactivated RSV (FI-RSV) induced serum IgG that inhibited viral shedding upon challenge, but also induced Th2-skewed responses. In cotton rats, similar effects of incorporation of MPLA in virosomes were observed with respect to induction of systemic antibodies and inhibition of lung viral shedding upon challenge, but mucosal sS-IgA responses were only moderately enhanced. Importantly, IN immunization with RSV-MPLA virosomes, like live virus infection, did not lead to any signs of ERD upon live virus challenge of vaccinated animals, whereas IM immunization with FI-RSV did induce severe lung immunopathology under otherwise comparable conditions. Taken together, these data show that mucosally administered RSV-MPLA virosomes hold promise for a safe and effective vaccine against RSV.

1. Introduction

Respiratory syncytial virus (RSV) infection causes viral bronchiolitis in infants and young children but also significant health problem in the elderly and immune-compromised individuals [1–3]. RSV infection at young age does not lead to life-long protection and multiple reinfections occur throughout life [2,4,5]. Vaccination of risk groups would be an effective approach to reduce the burden of disease. Although RSV has been recognized as an important vaccine target, no vaccine is available. This is, in part, due to the fact that immunization with inactivated RSV formulations or purified protein preparations can prime for enhanced respiratory disease (ERD) upon natural infection [6], as did a formalin-inactivated RSV vaccine (FI-RSV), evaluated in young children in the 1960s [7–9]. Hallmarks of ERD are neutrophilic alveolar infiltrates as well as perivascular and peribronchial infiltration of lymphocytes [10]. Immunization with FI-RSV also led to the induction of poorly neutralizing antibodies [11,12] as a result of impaired affinity maturation, probably because of a lack of Toll-like Receptor (TLR) signaling by FI-RSV [13]. Subsequent work in animal models showed that FI-RSV also induces Th2-skewed immune responses, as opposed to Th1-type responses that are better suited to protect against viral infections [14]. An approach to induce better neutralizing antibodies and Th1-skewed responses and to avoid priming

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for ERD, is to incorporate TLR ligands as immunomodulators in candidate non-replicating RSV vaccines [15].

RSV enters through the mucosal surface of the respiratory tract. A desirable feature of RSV vaccines would therefore be the capacity to induce, besides systemic antibody responses, also local immunity against RSV like secretory IgA antibodies (S-IgA). Mucosal immunization, through intranasal (IN) administration, could achieve such responses. It not only is a non-invasive and highly acceptable route of administration [16], in addition, it does not readily prime for enhanced disease, at least in animal models [17]. However, as mucosal surfaces are continuously exposed to antigens, mucosal immune tolerance mechanisms prevent untoward immune reactions. Therefore, inclusion of TLR ligands in an IN-administered RSV vaccine may well represent an essential prerequisite for induction of robust RSV-specific mucosal as well as systemic antibody responses [18]

A TLR ligand currently used in two registered intramuscular (IM) human vaccines is monophosphoryl lipid A (MPLA) [19,20]. We found that MPLA in RSV virosomes induces safe and protective immune responses in mice and cotton rats upon IM injection [21]. Interestingly, MPLA has also been reported to have adjuvant activity when co-administered IN with different vaccine antigens [22,23]. These findings therefore prompted further exploration of our candidate MPLA-adjuvanted RSV virosomal vaccine for induction of RSV-specific immunity upon IN administration.

Incorporation of MPLA in RSV virosomes administered IN to mice potentiated protective RSV-specific serum IgG and respiratory tract S-IgA antibody responses and induced Th1-skewed T cell responses. Incorporation of MPLA in RSV virosomes administered IN to cotton rats significantly increased virus-neutralizing serum IgG responses and protection against infection but only moderately stimulated mucosal S-IgA responses. In contrast to IM injection of FI-RSV, IN administration of RSV-MPLA virosomes did not prime for lung immunopathology upon challenge. These data combined show that mucosally administered RSV-MPLA virosomes hold promise for induction of protective immunity without priming for enhanced disease.

2. Materials and methods

2.1. Ethical statement

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

2.2. Virus

RSV 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) and purified as described before [21].

2.3. Vaccine formulations

RSV virosomes were generated as described previously [24]. Briefly, RSV membranes were dissolved in 100 mM 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC) in HNE (5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7.4) 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, monophosphoryl lipid A 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 min 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. After dialysis, virosomes were kept at 4°C. FI-RSV was produced as reported before [10].

2.4. Mouse immunization and challenge experiments

Female BALB/c OlaHsd mice (6–8 weeks old) were purchased from Harlan (Zeist, The Netherlands). For immunization and challenge, mice were anesthetized using isoflurane. Mice received RSV(-MPLA) virosomes (5 μg viral protein) IN in 50 μl HNE. Control mice received 25 μl of FI-RSV (5 μg viral protein) IM, 50 μl (10^6 $TCID_{50}$) of live RSV IN or 50 μl of HNE IN. Using this procedure, part of the IN inoculated volume may distribute further down to the lower respiratory tract. Vaccinations were given on day 0 and day 14 and on day 28 mice were challenged with 10^6 $TCID_{50}$ of live RSV IN, in a similar setup as a previous study on RSV-MPLA virosomes injected IM in mice [21]. On time points of vaccination and challenge, blood was drawn by retro-orbital puncture. Four days after challenge, mice were sacrificed and blood was sampled. Nose washes and broncho-alveolar lavages were done by incising the trachea and flushing of 1 ml PBS with protease inhibitors (Roche, Mannheim, Germany). Spleens were harvested for analysis of RSV-specific T cell cytokine responses and lungs for analysis of viral titers.

2.5. Cotton rat immunization and challenge experiments

Female outbred cotton rats (Hsd:Cotton Rat) of 4–6 weeks old were obtained from Harlan (Indianapolis, IN, USA). Rats received RSV(-MPLA) virosomes IN (5 μg viral protein). Control rats received 100 μl live virus (10^6 $TCID_{50}$) IN, 100 μl of HNE IN or 50 μl (5 μg viral protein) of FI-RSV IM. Vaccinations were given on day 0 and day 21 and on day 49, cotton rats were challenged with 10^6 $TCID_{50}$ RSV IN, in a similar setup as a previous study on RSV-Pam_3CSK_4 virosomes injected IM in cotton rats [24]. At the time of immunization and challenge, blood was drawn by retro-orbital puncture. Five days after challenge, rats were sacrificed and blood was sampled. Lung and nose washes were performed using similar techniques as in mice. Subsequently, the lungs were removed aseptically and one of the primary bronchi was ligated just below the tracheal bifurcation with suture wire. Half of the lung was kept on ice in HEp-2 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.

2.6. Immunological assays

RSV-specific antibody titers were determined as described before [24]. Briefly, 96-well plates were coated with betapropiolactone-inactivated RSV and blocked with 2.5% milk powder in coating buffer. Plates were incubated for 90 min with two-fold serial dilutions of serum or broncho-alveolar lavages, starting at dilutions of 1:200 for serum or 1:1 for BAL or nose washes. After washing, plates were incubated with a 1:5000 dilution of horseradish-peroxidase-coupled goat anti-mouse IgG, or IgA which bind to both mouse and cotton rat IgG and IgA, respectively (Southern Biotech 1030-05, 1040-05) for 1 h and subsequently stained with *o*-Phenylenediamine (OPD; Sigma-Aldrich,

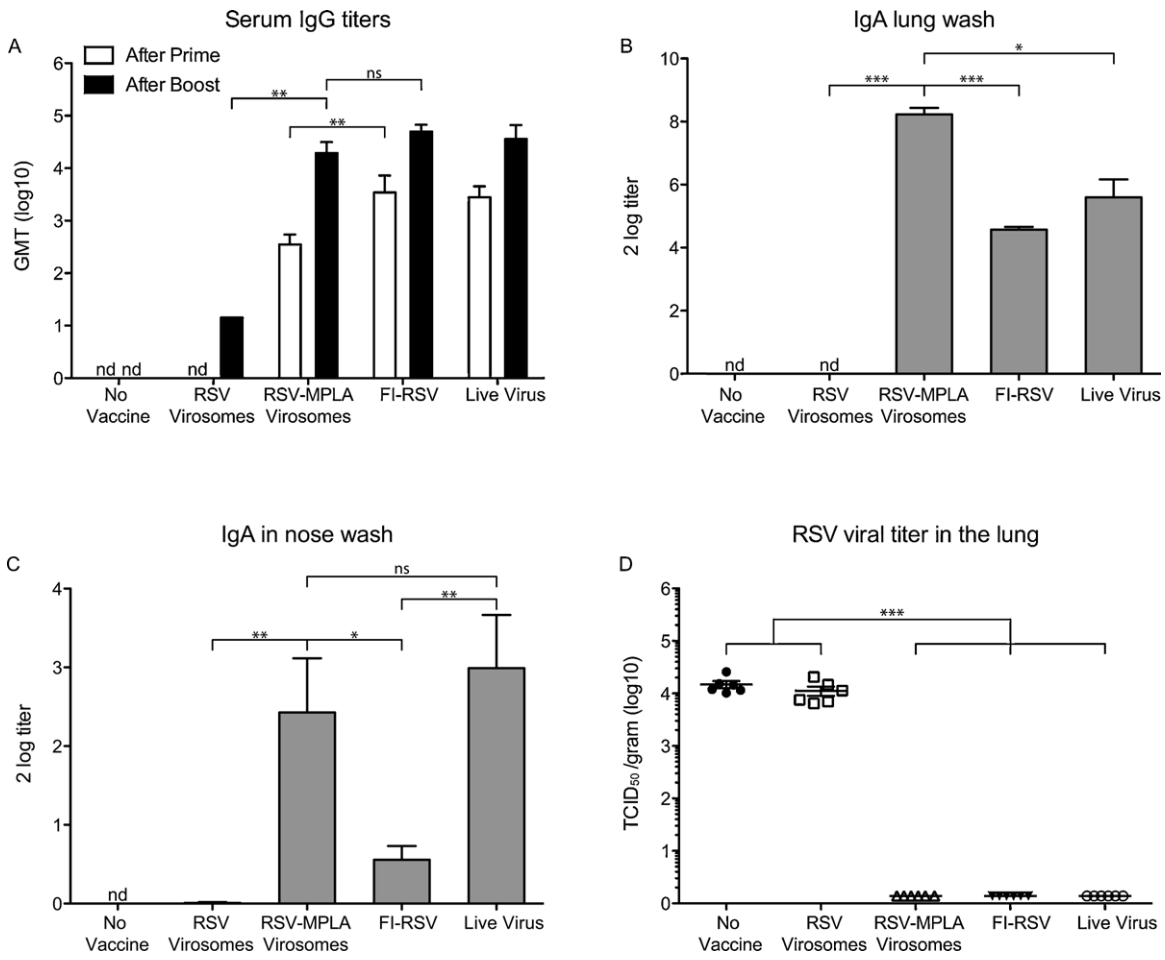

Fig. 1. Immunogenicity and protection in mice. Mice were immunized twice ("prime" on day 0 and "boost" on day 14) with RSV virosomes IN, RSV-MPLA virosomes IN, FI-RSV IM, and live virus IN. Control mice received buffer IN. Fourteen days after the immunizations blood was drawn and RSV-specific IgG in serum was determined (A). The immunized mice were challenged on day 28 with 10^6 $TCID_{50}$ RSV and terminated 5 days later. After termination IgA titers were determined in lung wash (B) and nose wash (C). RSV virus titers were determined by $TCID_{50}$ on lung homogenates. (Mann–Whitney *U* test: ns not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars represent the geometric mean titer ± SD (Panels A-C). Horizontal lines represent mean $TCID_{50}$ values (Panel D).

St Louis, MO, USA). After 30 min the staining was stopped by addition of 2 M H_2SO_4 and absorption was measured at 492 nm.

IFN-χ and IL-5 secretion in splenocyte cultures that were re-stimulated with inactivated RSV particles were assessed as described before [21].

2.7. Virus titration and microneutralization assay

Virus titers were determined by $TCID_{50}$ as described previously [21]. For determination of RSV virus neutralization titers, serum was decomplemented by heat inactivation for 30 min at 56°C. Neutralization titers were determined by incubation of two-fold serially diluted decomplemented serum with 70 $TCID_{50}$ of RSV for 2 h and subsequent titration of this mixture on HEp-2 cells as described before [21]. The neutralization titer was calculated with the Reed & Muench method as the dilution that neutralizes infection in 50% of the wells.

2.8. Histopathology

The inflated cotton rat lungs were embedded in paraffin and 4 μm slices were cut. The slides were stained with hematoxylin and eosin (H&E) using standard procedures. Subsequently, perivasculitis, peribronchiolitis and alveolitis were assessed by light-microscopy. Histopathology was assessed in more than one experiment and always included at least 3 animals per group.

2.9. Statistical analysis

Statistical analyses were performed with Graphpad Prism 5.00 for Mac OSX, (GraphPad Software, San Diego California USA, www.graphpad.com) using a Mann–Whitney *U* test. P values of 0.05 or lower were considered to represent significant differences.

3. Results

3.1. Immunogenicity in mice

To determine the adjuvant effect of MPLA in IN-administered RSV virosomes, we immunized mice with RSV virosomes or RSV-MPLA virosomes. RSV-naïve mice and mice immunized with FI-RSV or live virus, served as controls. RSV-MPLA virosomes, but not virosomes without MPLA, induced RSV-specific serum IgG, although levels were significantly lower than those induced by IM injection with FI-RSV or live RSV infection (Fig. 1A). One of six mice receiving a second IN immunization with RSV virosomes developed detectable RSV-specific serum IgG antibodies. In contrast, all mice that received a second IN immunization with RSV-MPLA virosomes

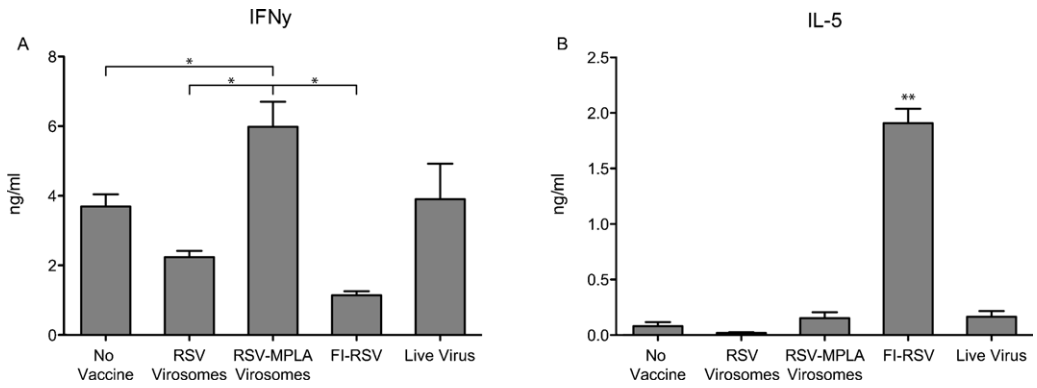

Fig. 2. Cellular immune response in mice. Mice were immunized, challenged and terminated as in Fig. 1. After termination, spleens were harvested and splenocytes were restimulated in vitro with BPL-inactivated RSV for three days. After three days IFN-γ (A) and IL-5 (B) were determined in the supernatants. (Mann–Whitney *U* test:,$^{*}p < 0.05$, $^{***}p < 0.001$). Bars represent mean cytokine levels ± SD.

developed IgG antibodies, to similar levels as in mice that received a second immunization with FI-RSV or a live virus infection (Fig. 1A).

For assessment of local immune responses, we analyzed lung and nose wash RSV-specific IgA antibodies. Mice immunized twice IN with RSV-MPLA virosomes showed significantly higher S-IgA levels in lungs compared to mice immunized with non-adjuvanted virosomes, FI-RSV or live virus infection. Both RSV-MPLA virosomes and live virus infection induced significantly higher nasal S-IgA compared to levels induced by non-adjuvanted virosomes and FI-RSV (Fig. 1B,C).

To determine protection against infection, immunized mice were infected with live RSV. Non-vaccinated mice or mice immunized IN with non-adjuvanted virosomes showed virus titers of approximately 10^4 $TCID_{50}$, 4 days post-infection. On the other hand, animals immunized with RSV-MPLA virosomes, FI-RSV or live virus had no detectable lung virus titers (Fig. 1D).

3.2. Cellular immune response in mice

Next, we determined Th1-type cytokine (IFN-ɣ) and Th2-type cytokine (IL-5) levels in RSV-restimulated splenocytes from immunized and subsequently challenged animals of all groups (Fig. 2). IFN-χ production in splenocyte cultures from mice immunized with RSV-MPLA virosomes was significantly higher than that in cultures from mice immunized with non-adjuvanted virosomes or FI-RSV. In contrast, IL-5 production in splenocyte cultures from mice immunized with FI-RSV was significantly higher than that in cultures from mice immunized with non-adjuvanted RSV virosomes, RSV-MPLA virosomes or live virus infection. Therefore, RSV-MPLA virosomes induced more Th1-skewed responses compared to responses induced by FI-RSV.

3.3. Immunogenicity in cotton rats

Next, we evaluated immune responses, protection and vaccine-induced immunopathology in the cotton rat model. Cotton rats, compared to mice, are more permissive to RSV and more prone to develop ERD. Similar to antibody responses in mice, low levels of RSV-specific serum IgG were detected in cotton rats immunized IN with non-adjuvanted RSV virosomes (Fig. 3A). However, incorporation of MPLA in the IN-administered virosomes significantly increased systemic IgG levels. Animals immunized once with RSV-MPLA virosomes showed significantly lower titers compared to those in cotton rats immunized once with FI-RSV or live virus. However, serum IgG antibody levels increased after the booster immunization to similar levels as seen in cotton rats primed and boosted with FI-RSV or live virus (Fig. 3A).

Next, the virus-neutralizing capacity of the sera were assessed. Sera from RSV-naïve cotton rats or rats immunized IN with non-adjuvanted RSV virosomes, did not have any significant neutralizing capacity (Fig. 3B). RSV-MPLA virosomes induced significantly increased levels of neutralizing antibodies compared to RSV virosomes without MPLA. These levels were, on average, also higher than those induced by IM immunization with FI-RSV, although the difference did not reach statistical significance. Live RSV infection, however, induced significantly higher neutralizing antibody levels compared to those induced by RSV-MPLA virosomes, administered IN, or FI-RSV, injected IM.

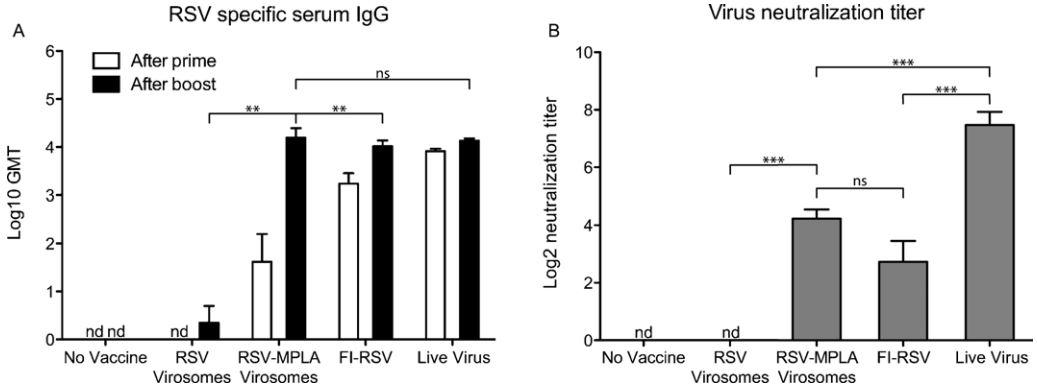

Fig. 3. Immunogenicity in cotton rats. Cotton rats were immunized with the same preparations as given to mice (Fig. 1) on day 0 and 21. On day 21 and 49, blood was taken en RSV specific IgG was determined in serum (A). RSV-virus neutralizing antibodies were determined in the day 49 serum (B). (Mann–Whitney *U* test: ns not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars represent the geometric mean titer ± SD.

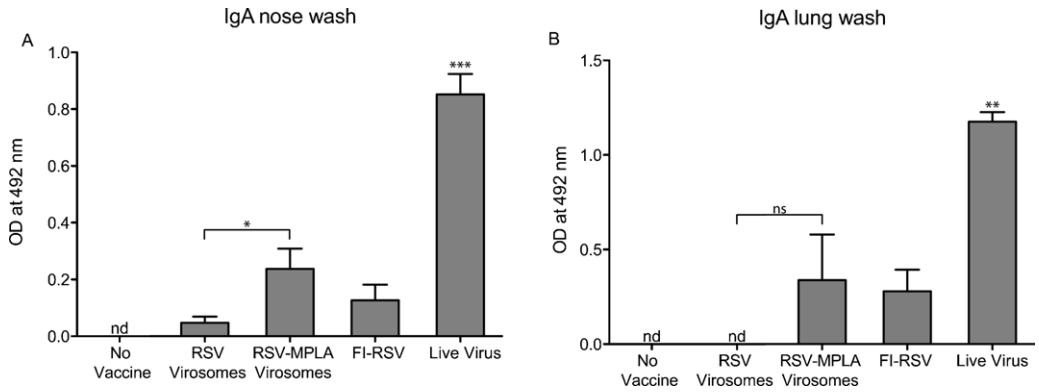

Fig. 4. Mucosal immune response in cotton rats. Cotton rats were immunized as in Fig. 3 and challenged with 10^6 $TCID_{50}$ live RSV on day 49. Five days after challenge, the rats were s acrificed and lung and nose washes were taken. RSV specific IgA was determined in nose washes (A) and lung washes (B). (Mann–Whitney *U* test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Bars represent mean OD values ± SD of 1:1 diluted washes.

Finally, the local antibody responses were determined. Although MPLA increased nasal S-IgA levels, levels of S-IgA in nose and lung induced by live virus infection were significantly higher than those observed in the other groups (Fig. 4)

3.4. Protection from RSV challenge in cotton rats

To determine protection against infection, immunized cotton rats were infected with live RSV. Significant lung virus titers were detected in RSV-naïve cotton rats and cotton rats immunized IN with non-adjuvanted RSV virosomes (Fig. 5A). Five out of seven cotton rats immunized IN with RSV-MPLA virosomes showed no lung virus titers, while two cotton rats had detectable virus titers, but at levels that were significantly lower compared to those in animals immunized IN with non-adjuvanted RSV virosomes. All animals immunized with FI-RSV or live virus had non-detectable lung viral shedding (Fig. 5A).

4. Histopathology analyses

To evaluate the safety of IN administration of RSV virosomes and RSV-MPLA virosomes, we harvested the lungs of immunized and challenged cotton rats and assessed lung pathology by light microscopy. Lungs from cotton rats immunized IM with FI-RSV showed clear signs of ERD, with perivascular and peribronchial infiltration and alveolitis with influx of predominantly neutrophils (Fig. 6 C,D). The lungs of non-immunized animals (Fig. 6 A,B), animals immunized IN with RSV virosomes (Fig. 6 E,F), RSV-MPLA virosomes (Fig. 6 G,H) or live virus (Fig. 6 I,J), however, did not show signs of ERD. Finally, immunization with FI-RSV, but not with RSV(MPLA) virosomes or live virus, lead to high neutrophil influx upon challenge of immunized animals (Fig. 7). This confirms the occurrence of immunopathology in the FI-RSV-immunized group and absence of this complication in animals immunized IN with RSV virosomes, RSV-MPLA virosomes or live virus.

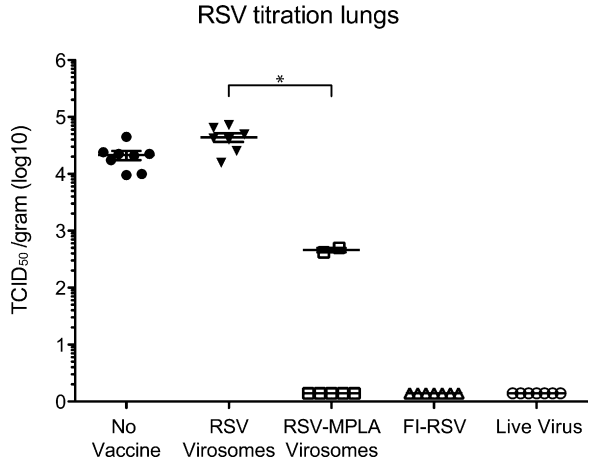

Fig. 5. Protection in cotton rats. Cotton rats were immunized, challenged and sacrificed as in Fig. 4. After termination, the lungs were removed and RSV titers in lungs were determined by $TCID_{50}$. (Mann–Whitney *U* test: ns not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Horizontal lines represent mean $TCID_{50}$ values.

5. Discussion

Intranasal administration represents an attractive route of administration for vaccines, including RSV vaccines. Effective induction of immune responses with non-replicating vaccine antigens through this route usually requires the use of adjuvants [25]. The adjuvant MPLA has an acceptable safety profile in humans and is currently being used in a number of licensed vaccines [26,27]. It does not only have immunomodulatory properties for induction of safe Th1-skewed responses against RSV [15,21], but also has been reported to have mucosal immunoadjuvant properties [22,23]. However, MPLA has not been tested before for its capacity to potentiate immune responses to a non-replicating RSV vaccine, such as RSV virosomes, upon IN administration. Here, we show that RSV virosomes with incorporated MPLA have the capacity to induce protective immune responses upon IN administration to mice and cotton rats, without priming for ERD.

IN administration of RSV-MPLA virosomes effectively induces serum IgG antibody responses and Th1-skewed immune responses, similar to RSV-MPLA virosomes administered by IM injection [21]. This is line with previous findings by others who compared the immunoadjuvant activity of MPLA co-administered IN or parenterally with antigen [28]. The adjuvant effect of MPLA is likely caused by the direct interaction of MPLA with TLR4 on dendritic cells (DC) that are abundantly present in draining lymph nodes, nasal or bronchus-associated lymphoid tissue, or even directly lining the respiratory tract. The activation leads to secretion of IL-12 and type 1 IFN, which skew T cell responses toward a Th1-phenotype [29]. Such responses may more safe with respect to the occurrence of ERD as they are likely to be associated with more safe Th1-skewed responses in RSV-infected lungs too, similar to responses we previously observed in mice immunized IN with inactivated RSV supplemented with TLR9/NOD2 ligands [18]. Similar to DC, B cells may be directly activated through TLR4 signaling which, together with aid of the induced T cell response, stimulates antibody responses [30]. Although many cell types in the respiratory

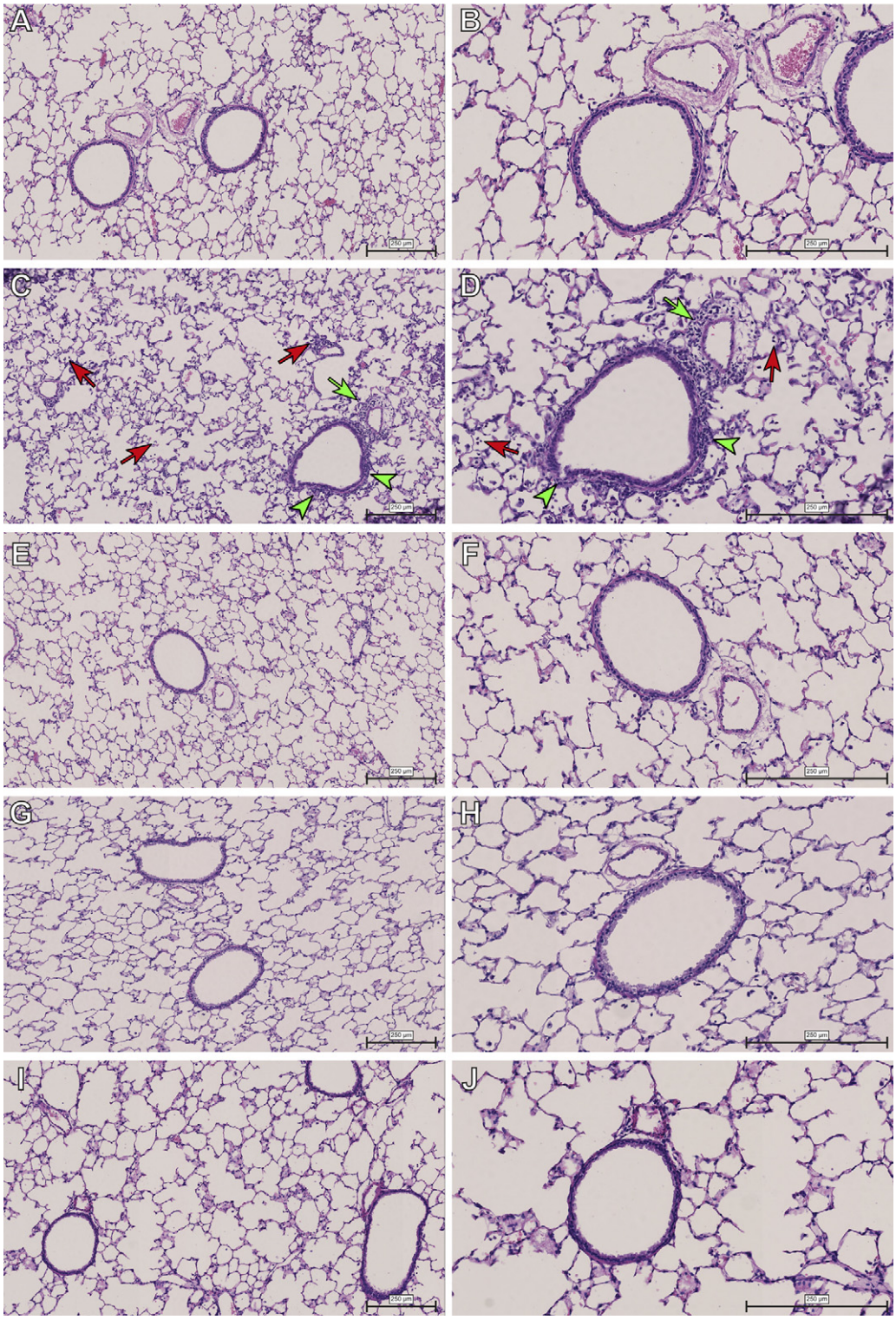

Fig. 6. Lung pathology in cotton rats. Cotton rats were immunized, challenged and sacrificed as in Fig. 4. After termination, one lung lobe was fixated with 4% formalin under 20 cm water pressure to retain the structure of the lung. After fixation, the lungs were embedded in paraffin and 4 μm slices were cut and stained with H&E. The lungs were evaluated by light microscopy. Left panels show lungs at a 200x magnification, right panels show lungs at a 400x magnification. Groups: No vaccine (A,B), FI-RSV (C,D), RSV virosomes (E,F), RSV-MPLA virosomes (G,H), Live virus (I,J). Red arrows, alveolar infiltrates, green arrows, perivascular infiltrates, green arrowheads, peribronchial infiltrates. The histopathology shown is representative of the histopathology observed in 3 animals per group.

mucosa express TLR4, the receptor for MPLA, the expression of the co-receptors CD14 and MD2, which are crucial for the initiation of TLR4-mediated cell signaling, are expressed at a lower level compared to their expression on, for instance, DC [31]. This reduced expression of CD14 and MD2 on the mucosal cell surfaces, e.g. epithelial cells, may reduce their susceptibility to endotoxins but possibly also to stimulatory effects of vaccine adjuvants such as MPLA [32]. This could explain the lower levels of RSV-neutralizing antibodies induced by IN immunization compared to IM immunization [21]. Other TLRs, such as TLR2 and TLR5, do not require

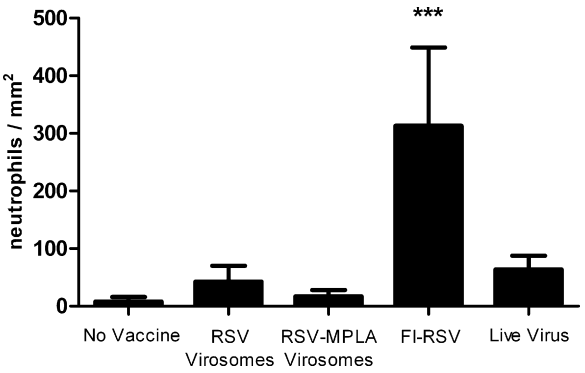

Fig. 7. Neutrophil infiltration in lungs of immunized and RSV-challenged cotton rats. Cotton rats were immunized, challenged and sacrificed as in Fig. 4. Lungs were removed, fixated and slices were stained with H&E. The numbers of infiltrating neutrophils were evaluated using light microscopy. Bars represent mean numbers ± SD.

these adaptor molecules and are also abundantly expressed on cells in the mucosal surfaces [31] and ligands for these receptors have been reported to have strong mucosal immunoadjuvant properties too [33,34]. How the mucosal immunoadjuvant activity of MPLA, co-administered in RSV virosomes, compares with that of other virosome-incorporated TLR ligands, such as a TLR2 ligand [24], remains to be investigated further.

Mucosal immunization can induce local S-IgA antibodies. More robust local S-IgA was induced by RSV infection in cotton rats, particularly when compared to responses induced by IN immunization with virosomes. In mice, differences in levels of S-IgA induced by infection or IN immunization were less pronounced. This difference may be related to the much higher permissiveness of the cotton rat for RSV infection than that of mice [35], leading to higher levels of viral replication and stronger local immune activation and, consequently, to higher S-IgA responses. Because FI-RSV also induces serum IgA (unpublished results), IgA found in washes of cotton rats or mice immunized with FI-RSV may originate from serum and translocate to the mucosa by transudation (in case of monomeric IgA) or through transcytosis mediated by the polymeric immunoglobulin receptor (pIgR; in case of polymeric IgA) [36]. Interestingly, TLR4 signaling upregulates expression of pIgR responsible for polymeric IgA transcytosis [36,37]. Clearly, RSV-specific serum IgG alone, for example induced by IM injection of RSV-MPLA virosomes, inhibits virus shedding in the lung. In this respect, we previously observed that lung viral titers negatively correlate with RSV-specific serum IgG levels [18], pointing to (sufficient levels of) serum IgG, as an important mediator of protection of the lungs. The upper respiratory tract, however, may not benefit so much from serum IgG for protection against infection, as transudation of antibody to this site is less efficient [38]. Rather, local S-IgA antibody may be more important for protection against viral infection at this site, as has previously been reported for influenza [39]. Further studies should clarify if S-IgA protects the upper respiratory tract by specifically analyzing nasal virus shedding in cotton rats immunized IN.

Together our data show that RSV-MPLA virosomes have the capacity to induce protective immunity upon IN administration to mice and cotton rats, without priming for enhanced disease. IN administration forms an attractive alternative to IM injection, as it is a non-invasive route of administration. Clearly, to potentiate RSV virosome-induced immune responses through this route, adjuvants are needed, which could be MPLA or possibly other TLR ligands with mucosal immunoadjuvant properties.

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References

[1] Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev 2010;23:74–98.
[2] Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med 2005;352:1749–59.
[3] Raboni SM, Nogueira MB, Tsuchiya LRV, Takahashi GA, Pereira LA, Pasquini R, Siqueira MM. Respiratory tract viral infections in bone marrow transplant patients. Transplantation 2003;76:142–6.
[4] Hall CB, Long CE, Schnabel KC. Respiratory syncytial virus infections in previously healthy working adults. Clin Infect Dis 2001;33:792–6.
[5] Falsey AR. Respiratory syncytial virus infection in adults. Semin Respir Crit Care Med 2007;28:171–81.
[6] Murphy BR, Sotnikov AV, Lawrence LA, Banks SM, Prince GA. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3-6 months after immunization. Vaccine 1990;8:497–502.
[7] Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89:422–34.
[8] Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. Am J Epidemiol 1969;89:449–63.
[9] Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am J Epidemiol 1969;89:435–48.
[10] Prince GA, Curtis SJ, Yim KC, Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. J Gen Virol 2001;82:2881–8.
[11] Murphy BR, Prince GA, Walsh EE, Kim HW, Parrott RH, Hemming VG, Rodriguez WJ, Chanock RM. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J Clin Microbiol 1986;24:197–202.
[12] Murphy BR, Walsh EE. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. J Clin Microbiol 1988;26:1595–7.
[13] Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, Diaz L, Trento A, Chang H-Y, Mitzner W, Ravetch J, Melero JA, Irusta PM, Polack FP. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15:34–41.
[14] Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J Virol 1996;70:2852–60.
[15] Boukhvalova MS, Prince GA, Soroush L, Harrigan DC, Vogel SN, The Blanco JCG. TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. Vaccine 2006;24:5027–35.
[16] Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006;58:68–89.
[17] Hussell T, Humphreys IR. Nasal vaccination induces protective immunity without immunopathology. Clin Exp Immunol 2002;130:359–62.
[18] Shafique M, Wilschut J, de Haan A. Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands. Vaccine 2012;30:597–606.
[19] Garçon N, Segal L, Tavares F, van Mechelen M. The safety evaluation of adjuvants during vaccine development: the AS04 experience. Vaccine 2011;29:4453–9.
[20] Garçon N, Wettendorff M, van Mechelen M. Role of AS04 in human papillomavirus vaccine: mode of action and clinical profile. Expert Opin Biol Ther 2011;11:667–77.
[21] Kamphuis T, Meijerhof T, Stegmann T, Lederhofer J, Wilschut J, de Haan A. Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid a in mice. PLoS ONE 2012;7:e36812.
[22] Childers N, Miller K, Tong G, Llarena J, Greenway T, Ulrich J, Michalek S. Adjuvant activity of monophosphoryl lipid A for nasal and oral immunization with soluble or liposome-associated antigen. Infect Immun 2000;68:5509–16.
[23] Baldridge JR, Yorgensen Y, Ward JR, Ulrich JT. Monophosphoryl lipid A enhances mucosal and systemic immunity to vaccine antigens following intranasal administration. Vaccine 2000;18:2416–25.
[24] Stegmann T, Kamphuis T, Meijerhof T, Goud E, de Haan A, Wilschut J. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. Vaccine 2010;28:5543–50.
[25] Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, Mills KHG, Rappuoli R, Del Giudice G. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. Vaccine 2001;19:2534–41.

[26] Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. Immunol Rev 2011;239:178–96.
[27] Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, Kielland A, Vosters O, Vanderheyde N, Schiavetti F, Larocque D, van Mechelen M, Garçon NAS04. an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 2009;183:6186–97.
[28] Sasaki S, Hamajima K, Fukushima J, Ihata A, Ishii N, Gorai I, Hirahara F, Mohri H, Okuda K. Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine. Infect Immun 1998;66:823–6.
[29] Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 2007;316:1628–32.
[30] Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, Marsland BJ. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. J Immunol 2007;178:2182–91.
[31] Parker D, Prince A. Innate immunity in the respiratory epithelium. Am J Respir Cell Mol Biol 2011;45:189–201.
[32] Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. Am J Physiol Lung Cell Mol Physiol 2004;287:L428–37.
[33] Ainai A, Ichinohe T, Tamura S-I, Kurata T, Sata T, Tashiro M, Hasegawa H. Zymosan enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine. J Med Virol 2010;82:476–84.
[34] Cataldi A, Yevsa T, Vilte DA, Schulze K, Castro-Parodi M, Larzábal M, Ibarra C, Mercado EC, Guzman CA. Efficient immune responses against Intimin and EspB of enterohaemorragic Escherichia coli after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. Vaccine 2008;26:5662–7.
[35] Boukhvalova MS, Prince GA, Blanco JCG. The cotton rat model of respiratory viral infections. Biologicals 2009;37:152–9.
[36] Johansen F-E, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. Mucosal Immunol 2011;4:598–602.
[37] Bruno MEC, Frantz AL, Rogier EW, Johansen F-E, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF-(B pathways in intestinal epithelial cells. Mucosal Immunol 2011;4:468–78.
[38] Renegar K, Small P, Boykins L, Wright P. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. J Immunol 2004;173:1978–86.
[39] Renegar KB, Immunoglobulin Small PA. A mediation of murine nasal anti-influenza virus immunity. J Virol 1991;65:2146–8.