Development of a mucosal vaccine against Respiratory Syncytial Virus infection

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Dedicated to

Whose Eternal Prayers
Always Paved the Path to my Success

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Chapter 1

General introduction

Introduction

Respiratory syncytial virus (RSV) infection is a major cause of lower respiratory tract disease among infants, young children and immunocompromised individuals. Natural infection with RSV results in incomplete immunity and reinfection may occur frequently throughout life. For this reason, RSV infection forms a serious threat in chronically ill adults and the elderly [1]. Recent studies have demonstrated that RSV also makes a major contribution to mortality among the elderly, indeed to similar extents as does influenza [2]. Presently, the only approved medication against RSV infection is a prophylactic monoclonal antibody, i.e. Palivizumab, which is given as a prophylaxis to high-risk infants. Despite the identification and isolation of the virus in 1956, efforts to develop a safe vaccine have been unsuccessful so far. In a clinical trial conducted in young children in the 1960s, a formalin-inactivated RSV (FI-RSV) vaccine did not protect against infection, but rather caused enhanced respiratory disease (ERD) upon subsequent exposure of the vaccinees to the natural virus. The finding that inactivated RSV vaccines may prime for ERD has contributed significantly to the delay of their further development. With the recent identification of new-generation adjuvants and immunomodulators, such as innate receptor ligands, the interest in inactivated RSV vaccine development has renewed. An attractive approach to the development of a safe and effective RSV vaccine, may be the use of innate receptor ligands as adjuvants in a mucosally (e.g. intranasally; IN) administered RSV vaccine.

Respiratory Syncytial Virus

RSV is an enveloped negative-sense non-segmented single-stranded (ss) RNA virus of belonging to the Pneumovirus genus of the family Paramyxoviridae. This family also comprises other major human pathogens such as Measles, Mumps and parainfluenza virus [3]. Two serotypes of RSV have been recognized, i.e. RSV A and RSV B [4].

The RSV genome contains 10 genes of 15,200 nucleotides encoding 11 proteins [5]. RSV consists of a nucleocapsid surrounded by a lipid envelope with a diameter of 150-300 nm (Figure 1: RSV particle and RSV-genome). RSV expresses two non-structural proteins, NS1 and NS2. These are detected only in RSV-infected cells and are not packaged into the virion. They mainly serve to inhibit type I interferon responses [6]. Eight RSV proteins are present in the virion particles. Among these structural proteins, three are membrane proteins: the attachment protein G, the fusion protein F and the small hydrophobic protein (SH). The heavily glycosylated G protein is responsible for viral attachment to the cell. The F protein not only contributes to binding of the virus to cells, but also plays a crucial role during virus entry by mediating fusion of the viral envelope with the cell membrane, thereby allowing deposition of the viral genome into the cytosol

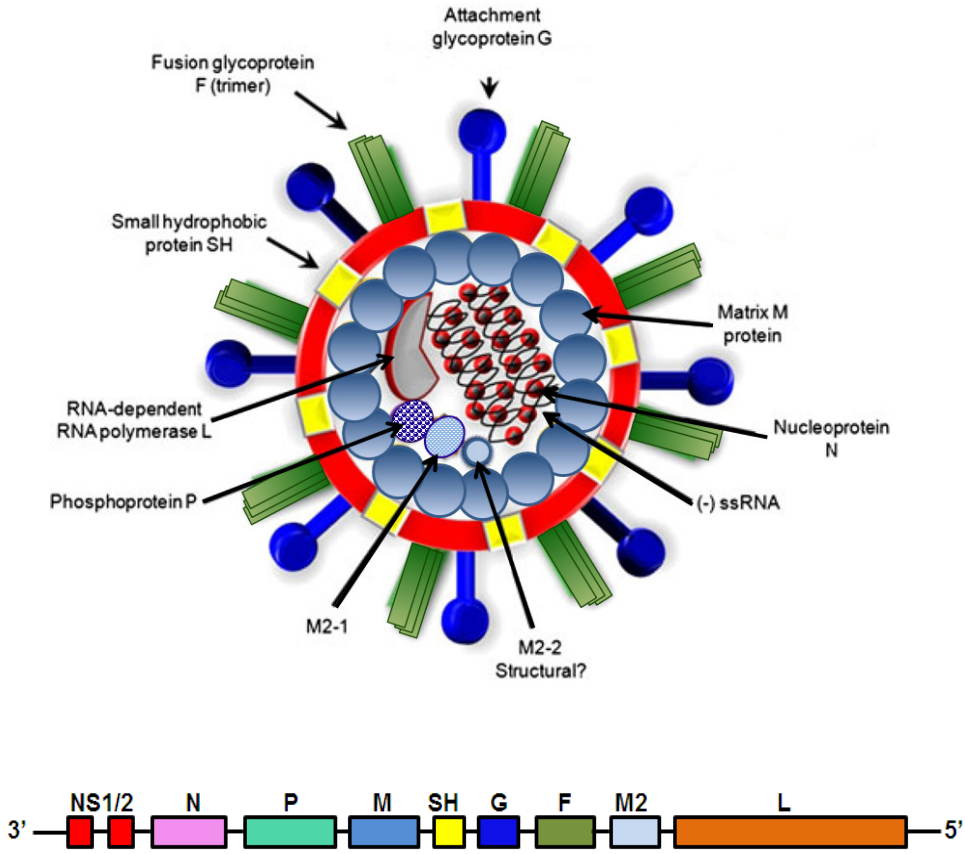

Figure 1: Schematic representation of the RSV virion and genome composition (adapted from [9])

[7]. Besides this, the F protein is a mediator of syncytium formation [5]. The function of the SH protein, which is predominantly found in the infected-cell membrane, is unknown [8]. The remaining viral structural proteins are the matrix protein (M), the nucleocapsid protein (N), the RNA-dependent RNA polymerase (L), the phosphoprotein (P) and the M2 gene product M2-1: all these proteins reside inside the viral particle. Whether the M2-2 gene product is packaged in the virion is currently unknown [9].

The function of the matrix (M) protein is to connect the viral nucleocapsid with the lipid envelopes and it is also responsible for viral particle assembly. The M2-2 protein is involved in regulation of viral transcription [10]. M2-1 functions as transcription-elongation factor [11]. The nucleocapsid protein (N) and phosphoprotein (P) are essential for transcriptional activity, while the L protein has RNA polymerase activity.

Epidemiology

RSV infections have a world-wide distribution. In temperate climates, infections are confined to the winter season. In tropical climates, RSV infections may occur throughout the year, but can be more frequent in the rainy season in some geographical areas [12-14]. Although infection can be established in several laboratory animals, natural infection with RSV seems to be limited to humans and apes [15]. RSV transmission occurs through direct contact or contact with contaminated surfaces that harbor respiratory secretions. The virus can survive for several hours on toys or other objects, which explains the high rate of nosocomial RSV infections particularly in pediatric wards. The incubation period for RSV infection ranges between 2 and 7 days [16]. Almost 70% of newborns are infected in the first year of their life. By 2 years of age almost all children have been infected and over 50% will have been infected twice [17]. RSV infections are common in the population and re-infections probably occur frequently. In a study conducted by Hall *et al.*, almost 25% of adult volunteers could be re-infected with RSV of the same group, two months after a natural infection [18].

RSV infection is the most important cause of severe respiratory illness in infants and young children and is the most frequent cause of hospitalization of infants and young children in industrialized countries [19]. RSV infections differ in disease severity, ranging from a mild cold to bronchiolitis or pneumonia. Approximately 3% of infants infected with RSV requires hospitalization due to respiratory failure and feeding problems [20]. Among hospitalized infants, 20% needs mechanical ventilation [21]. The highest morbidity of RSV disease is seen in children below the age of 6 months [22] and in children with underlying risk factors such as prematurity [23], broncho-pulmonary dysplasia [24], congenital heart disease with increased pulmonary circulation [25] or immune deficiency [26]. According to the WHO, the global RSV disease burden is estimated at 64 million cases and 160,000 deaths each year. In USA alone, approximately 85,000 to 144,000 infants are admitted to hospitals with RSV infection per year, which corresponds to 20-25% of pneumonia cases and up to 70% bronchiolitis cases in the hospital [27,28]. The elderly people are also at risk for severe RSV disease and 14,000 to 62,000 RSV-associated hospitalizations of the elderly occur in the USA with an estimated annual cost of RSV pneumonia-related hospitalizations of $150-680 million [29,30].

Pathogenesis

After infection, RSV primarily replicates in the epithelial cells of the nasopharynx [31]. The exact mechanism by which RSV spreads to the lower respiratory tract is unknown. Presently, it is not clear why the disease course is mild in most children, but severe in a small subgroup. Different studies have described associations between disease severity and genes involved in allergic responses, like IL-4 and IL-4 receptor genes, and genes for inflammatory cytokines, e.g. IL-6 and IL-8 [32]. Furthermore, up-regulation

of chemokines during RSV infection is associated with disease severity. For example, CCL11 (eotaxin), RANTES (CCL5) and MIP-1α have been found in higher levels in cases with more severe RSV infection and ERD [33,34].

Several other factors could be associated with disease severity, including, for example, environmental factors, patient intrinsic factors, virus strain type and viral load. Environmental factors like a high number of siblings, attendance of day-care centers and socio-economic status can enhance the chance of early exposure and may increase the risk of developing lower respiratory tract disease [35]. Other factors like geographical area, parental smoking and the use of wood-burning stoves have also been linked to an enhanced risk of severe RSV infections [36-39]. Patient-intrinsic factors like a compromised respiratory function, e.g. bronchopulmonary dysplasia (BPD) [40], or congenital heart disease with increased pulmonary circulation may significantly enhance the risk to develop severe RSV infection [41]. It is reported in some studies that RSV-strain A is responsible for more severe disease [42], while other studies report no difference between RSV A and B strains [43,44]. Furthermore, the course of lower respiratory disease was found to be associated with a high viral load [45]. Finally, RSV-specific immunity induced by vaccination may also be involved in immunopathological mechanisms leading to enhanced disease. This hypothesis is mainly based on experimental animal data [46], and on observations from a clinical trial where, as indicated above, infants were vaccinated with a formalin-inactivated candidate vaccine (FI-RSV), which resulted in ERD upon natural infection [47-49]. The notion that inactivated-RSV preparations can prime for ERD is one of the factors that has delayed the development of an effective RSV vaccine.

Immune responses after RSV infection

Innate immune responses

Innate immunity provides the first line of resistance to infectious diseases. During a natural RSV episode, the virus mainly infects airway epithelial cells from alveoli and small airways [50]. These epithelial cells, together with specialized dendritic cells (DC) and macrophages, are the first cells that respond to the infection [51]. For example, RSV can be recognized by three classes of pattern recognition receptors (PRRs), including Toll-Like Receptors (TLRs), NOD-Like Receptors (NLRs) and RIG-Like Receptors (RLRs). TLRs are found on a wide range of cells such as macrophages, DCs, epithelial cells, eosinophils and neutrophils. A number of TLRs are activated during RSV infection, including TLR2, TLR3, TLR4 and TLR7 [52]. TLR2 is expressed on the surface of immune cells, but also on epithelial cells, mostly in conjunction with other receptors such as TLR1, TLR6, CD36, CD14 or dectin-1 [52]. Recent studies have indicated that TLR2 is involved in RSV recognition and subsequent innate immune activation and suggest that TLR2 may be a functional receptor for RSV [52]. TLR3 is an intracellular receptor that recognizes double-stranded RNA (dsRNA), which is produced during RSV

replication. TLR4 is activated on the cell surface upon binding of the RSV-F protein using CD14 as co-receptor [53,54]. Activation of TLR4 and CD14 by RSV-F protein leads to an NF-κB-mediated inflammatory response and innate immune responses [55] and increased TLR4 expression in epithelial cells [56]. RSV particles contain ssRNA that is being recognized via TLR7 after entry of the viral genome into the cell cytosol [52]. Furthermore, RSV infection may also stimulate NOD2 expression, which activates IRF3 and type-I IFN production within 2h post-infection, whereas other PRRs (e.g. RIG-I) activate the IRF3-IFN pathways later on during infection [57]. The latter pathways serve to induce a general antiviral state through production of type I IFNs. Innate immunity also primes the adaptive immune system in such a way that it can induce effective immune responses to clear the virus and also provide protection against reinfection [58].

Acquired cellular immunity

Virus-specific T cells ($CD4^+$ and $CD8^+$ T cells) play a prominent role in the clinical outcome of RSV infection. Previously, it has been described that in children with normal T cell responses, virus excretion stops within 1-3 weeks, while children who do not mount an adequate cellular immune response, may shed virus for prolonged periods of times [59]. Furthermore, patients with impaired T cell immunity display more severe disease and enhanced virus shedding [26]. RSV-induced respiratory disease in infants also correlates with decreased frequencies of T cells in the lungs of these children [60].

Role of $CD4^+$ T cells

T-helper (Th) cells are generally classified into Th1, Th2 and Th17 subsets on the basis of their cytokine secretion profiles. For example, Th1 cells are known to secrete IL-2, IFN-γ and TNF-α; Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 while Th17 cells secrete IL-17.[[61]. The general view is that an effective protection against RSV, for example, induced by infection or vaccination, relies on a virus-specific T cell responses skewed towards Th1-type response [46]. RSV has evolved mechanisms, however, that skew towards less effective Th2-type responses upon infection [62].

In human studies, contradictory observations have been made regarding the role of $CD4^+$ T cells. RSV infection in infants has been reported to lead to induction of higher levels of Th2-type immune responses (e.g. with IL-4 production) and lower levels of Th1-type responses (e.g. with IFN-γ production) [63]. It has also reported, however, that in young infants, IFN-γ is the predominant cytokine produced by T cells regardless of clinical severity [64]. In another study, an enhanced IFN-γ production was found in nasopharyngeal washes of children exhibiting severe disease symptoms compared to those with mild disease [65]. In contrast, yet another study demonstrated that RSV infection of infants younger than 3 months induced increased levels of IL-4 in nasopharyngeal washes, followed by marked eosinophilia and establishment of Th2-type responses that were associated with enhanced disease [66].

It has been reported that vaccination of mice with inactivated vaccines, including FI-RSV or RSV-G protein, elicited Th2-cytokine expression upon infection with live RSV [67-70]. Additionally, vaccination of mice or cotton rats with FI-RSV was linked with enhanced lung pathology characterized by infiltration of eosinophils [67,71], and this enhanced pathology was reduced by simultaneous administration of IL-4- or IL-10-specific monoclonal antibodies (mAb), but not by IFN-γ- or IL-2-specific mAb [72]. This suggests that Th2-type cytokines, but not Th1-type cytokines, are involved in enhanced disease.

Role of $CD8^+$ T cells
In general, $CD8^+$ T cells are thought to play a beneficial role in RSV-specific immunity [73]. $CD8^+$ T cell responses are mostly directed against NS2, N, M, M2, F and SH proteins, with responses against F, N and M2 being the most prominent in mice [74]. It has been reported that enhanced frequencies of RSV-specific $CD8^+$ T cells in infants correlate with a decreased risk for secondary RSV infection [75], may assist in clearance of the virus [76]. There is sufficient data to support the protective role of $CD8^+$ T cells in RSV infection. It has been observed that RSV-specific $CD8^+$ T cells were able to clear virus more rapidly than immunodeficient mice [77]. This has been further confirmed by cell transfer studies where RSV infection in immunodeficient mice was stopped by transfer of RSV-specific $CD8^+$ T cells [78]. Recent studies have also demonstrated the presence of RSV-specific $CD8^+$ T cells in the bronchial alveolar lavage and blood of RSV-infected infants [76]. In human studies, decreased clinical symptoms were observed with increased numbers of $CD8^+$ T cells in the peripheral blood of previously infected adults [79]. Despite the well-established role of $CD8^+$ T cells in clearing virus after RSV infection, some contribution of $CD8^+$ T cells to enhanced disease in mice has also been reported, for example, when excessive RSV-specific $CD8^+$ T cells were transferred to RSV-infected mice [80].

Acquired humoral immunity

Systemic IgG
After primary infection, IgG antibody responses follow the initial transient IgM response and reach peak titers at approximately 20-30 days post-infection, while, upon secondary infection, IgG responses are faster and higher in magnitude and reach peak titers in 5-7 days [81]. Serum IgG antibodies can transudate to the lower respiratory tract and the lungs, but transudation to the upper respiratory tract (i.e. nasal mucosa) is not efficient. It is, therefore, believed that the main function of serum IgG is to contribute to virus clearance from the lower airways and lungs [82,83]. In line with this, several studies in humans and animals indicate that RSV-specific serum IgG titers negatively correlate with disease severity and viral titers in the lower respiratory tract in young children as well as in the elderly and also in mice [84-86].

Local IgA
Secretory IgA (S-IgA) antibody responses are mainly associated with protection of mucosal surfaces. RSV replication occurs primarily in the respiratory epithelial cells that line the airways, so local S-IgA can form a first barrier against infection [87]. It has been reported that RSV-specific IgA is secreted rapidly in the upper airways following primary RSV infection in mice [88]. Despite the increase in IgA-secreting plasma cells after infection of mice, RSV-specific IgA levels appeared to wane fast over time, being largely undetectable by 8 weeks post–infection [89]. Reduced RSV-specific nasal IgA titers have been found with increased infection in human adults [84]. Murine studies have also demonstrated that intranasal (IN) administration of RSV-specific IgA monoclonal antibody prior to RSV infection could provide protection [90]. In mice, administration of IgA monoclonal antibody appears to be equally effective as IgG monoclonal antibody in providing protection against RSV infection [91]. Thus, S-IgA could play an important role in preventing RSV infection of the airways.

Treatment of RSV infection

Antibody prophylaxis

RespiGam was the first antibody preparation which could reduce disease severity in high-risk infants [92]. A major drawback of its use was the requirement of the product as a large-volume intravenous infusion. With advances in neutralizing monoclonal antibody technology, this approach was further refined by the development of Palivizumab (Synagis), a humanized monoclonal antibody that recognizes an epitope on the RSV-F protein. Palivizumab is 50- to 100-fold more potent than RespiGam and can be administered by intramuscular injection [93]. Presently, palivizumab is the only approved monoclonal antibody for the prophylaxis of RSV. It reduces RSV-related hospitalizations by 55% [94]. It, however, has little effect on the replication of the virus in the upper respiratory tract of infants [93]. Motavizumab is an improved form of Palivizumab which elicited 20-fold enhanced neutralization of RSV *in vitro* compared to Palivizumab and potently inhibits RSV replication in the upper respiratory tract of cotton rats [95]. It has, as yet, not been registered for clinical use.

Antivirals and other treatment modalities

Despite the success in development of prophylactic antibodies, as described above, some antiviral drugs are also being used in high-risk infants or in immunocompromised individuals. Ribavirin (a nucleoside analogue), is the only approved drug to treat RSV infections, but there is extensive debate about its clinical benefits in young children [96]. In addition to this classical antiviral treatment modality, new approaches involving like small interfering RNAs (siRNAs) have been explored. These approaches reduce viral protein production by inhibiting targeted mRNAs in a sequence-specific manner.

IN administration of siRNAs prior to infection, for example, inhibited RSV replication in mice [97,98].

Vaccination against RSV

Despite more than four decades of research, a safe and effective RSV vaccine is not available. Attempts to develop an formalin-inactivated RSV vaccine were initiated in the 1960s, but with disastrous outcome as discussed in more detail below. Several approaches like the use of live-attenuated RSV, subunit or purified proteins, chimeric viruses and vector-based vaccines, have been or are still under development as candidate RSV vaccination strategies. Most of these approaches have been tested in clinical trials but none of them reached the required safety and efficacy standards, and demonstrated a variable degree of immunogenicity, stability and safety. A number of RSV vaccine candidates and challenges associated with their development are described below.

Formalin-inactivated vaccines

Attempts to control RSV infections in newborn and young children by vaccination were initiated in the 1960s. Four studies were performed in young children who received formalin-inactivated whole RSV virus (FI-RSV) vaccine [47-49,99]. These studies demonstrated that FI-RSV vaccine failed to protect upon subsequent natural infection. Rather, in many cases ERD was observed. In one of these studies, 80% of vaccinees required hospitalization, compared to only 5% of vaccinees who received a parainfluenza vaccine. Of the hospitalized children suffering from ERD, two individuals died. Upon natural virus infection, FI-RSV vaccinees experienced severe symptoms like pneumonia and/or bronchiolitis, while children from control groups experienced more mild symptoms like rhinitis, pharyngitis and/or bronchitis [47]. Autopsy reports from the deceased children describe lung infiltrates consisting mostly of neutrophils and eosinophils, indicating a role of host inflammatory responses in the enhancement of disease [100]. Further analysis of sera from FI-RSV vaccinees showed that the vaccine had induced poorly virus-neutralizing antibodies, which may have contributed to ERD by delaying virus clearance from the lungs [101].

After this disastrous vaccine trial outcome, several studies were carried out in animal models to explore the etiology of the observed pulmonary immunopathology. Possible factors involved in priming for ERD by FI-RSV include disruption of protective epitopes by chemical inactivation of the virus, poor innate receptor activation by the vaccine resulting in induction of poorly neutralizing antibodies, and excessive induction of Th2-type immune responses potentially due to the use of alum as an adjuvant [67,102-105]. Additionally, vaccine-induced IgE is believed to contribute to hypersensitivity responses associated with ERD, as demonstrated in calves immunized with formalin-inactivated-Bovine RSV (FI-BRSV) followed by BRSV challenge [106].

Live-attenuated vaccines

Live-attenuated (LA) virus vaccines have been considered an effective strategy because they mimic natural virus infection and probably, following the use of LA vaccines, no enhanced pathology is to be expected. If the vaccine is delivered via the mucosal route, a balanced systemic and local immune response may develop including the induction of mucosal antibodies and cytotoxic T cells, which are known to be essential in protection against infection and clearance of the virus. However, with attenuated RSV vaccines, the balance between immunogenicity and attenuation appears difficult to establish [73]. Inadequate attenuation and the potential reversion of the vaccine virus to a wild-type form pose serious risks in terms of induction of pathology and potential mortality, especially in immunocompromised individuals [107]. The first LA vaccine, *cpts*248/404, was evaluated in infants 1-2 months of age. Vaccination resulted in a high incidence of nasal congestion and the virus turned out to be insufficiently attenuated for use in very young children [108]. Mutated strains, like those lacking the SH gene, did not lead to nasal congestion in infants. However, the immunity conferred by this vaccine did not correlate with protection [109]. Currently, a new live-attenuated RSV strain (MEDI-559) is in Phase II clinical evaluation in children (5-24 months of age) and infants (1-3 months of age).

Subunit vaccines

A purified RSV-F protein (PFP-2 and PFP-3) subunit vaccine adjuvanted with alum has been tested in pregnant women, healthy adults over 60 years of age and children with cystic fibrosis (CF) [110-112]. The vaccine induced a ≥4-fold increase in virus-specific antibodies in 95% of the vaccinated pregnant women, and RSV-specific antibodies in the newborns and breast milk from vaccinated mothers were significantly enhanced [111]. Another subunit vaccine comprising purified RSV-F, -G and -M proteins, has been evaluated in a clinical dose-ranging study in 561 adults of 65 years or older. The vaccine's reactogenicity was similar to that seen with seasonal influenza virus vaccination. Unexpectedly, the non-adjuvanted vaccine appeared to be more immunogenic than the alum-adjuvanted vaccine and was the only formulation that induced a ≥4-fold rise in neutralizing antibody titers against RSV in ≥50% of the vaccinees [113]. Similar results were also found in another clinical trial in adults with cardiopulmonary disease, in which non-adjuvanted subunit vaccine was more immunogenic than an aluminum-phosphate-adjuvanted vaccine. This vaccine was found to be safe and immunogenic. However, RSV-specific antibody titers returned to baseline within a year, suggesting that annual vaccination would be required [114].

Chimeric viruses and virus-like particles

In this approach, selected RSV genes are expressed in related paramyxoviruses like Newcastle disease virus, bovine parainfluenza virus (PIV) or Sendai

virus that can be delivered to the respiratory tract. The advantage of using these viruses is that their replication is limited in humans as humans are not their natural host. A bovine PIV3 expressing the RSV-F and human PIV-F, (MEDI-534) is the only chimeric virus vaccine that has been evaluated in a clinical trial. This vaccine induced protection in African green monkeys upon challenge with wild-type RSV, however, the virus-neutralizing antibody levels were low [115]. This vaccine was also found safe and well tolerated in clinical trials with induction of low virus-neutralizing antibody in humans [116]. To further investigate the protective efficacy of this vaccine, a phase 1/2a clinical trial is in progress [117]. Another, version of this strategy involves the use of virus-like particles composed of the Newcastle disease virus (NDV) nucleocapsid and membrane proteins and chimeric proteins of RSV F and G proteins fused to the transmembrane and cytoplasmic domains of NDV F and HN proteins, respectively, were evaluated in mice. This VLP-based vaccine induced Th1-skewed virus neutralizing antibody titers which remained stable for 4 months in mice without leading to enhanced disease [118].

Replication-defective gene-based vectors

Among the several replication-defective vectors, only recombinant alphaviruses and adenoviruses (rAd) are presently being explored as vectors in RSV vaccines. Alphaviruses have been used to express several antigens, including antigens derived from influenza virus and cytomegalovirus (CMV) [119]. Alphavirus replicon particles derived from Venezuelan Equine Encephalitis virus (VEE) expressing the RSV-F (VRP-RSV-F) have been tested in mice and cotton rats after IN administration and have been shown to induce mucosal immune responses and provide protection against RSV infection [120]. Moreover, the VEE-based RSV vaccine did not induce Th2-skewed immune responses. Recently, an RSV vaccine based on a recombinant adenovirus (rAd) vector has been described as a potential platform for both adult and pediatric vaccination and evaluated in a preclinical trial [73].

A new approach to RSV vaccination

A general view is that LA vaccines would be most suitable for use in young children while inactivated (non-replicating) virus vaccines would be the preferred vaccine modality for use in adults and the elderly [107]. Non-replicating RSV vaccines produced from cultured virus should, however, avoid the use of chemicals (i.e. formalin) to inactivate the virus, as this could disrupt protective epitopes and lead to induction of non-neutralizing antibodies [103,121].

RSV virosomes are non-replicating particles and their production does not require harsh chemical treatment [122]. To potentiate antibody responses, a suitable adjuvant system should be chosen. As alum has been shown to have limited value when co-formulated with inactivated RSV vaccines [113], other adjuvants need to be explored. Innate receptor

ligands, like Toll-like receptor (TLR) ligands, have been shown to have great promise as vaccine adjuvants and are already in use in licensed vaccines (e.g. Cervarix). These types of adjuvants could improve the affinity of vaccine-induced RSV-specific antibodies and also skew cellular immune responses towards more effective and safe Th1-type responses [123].

Finally, mucosal administration could form an attractive approach to vaccination against RSV. It is a highly accepted and non-invasive route of immunization and also could induce mucosal antibody responses (i.e. S-IgA) that may contribute to protection. In this strategy, innate receptor ligands, like TLR ligands, could be employed as they have shown to possess mucosal immunoadjuvant activity [124]. Therefore, mucosal immunization with non-replicating RSV particles, like RSV virosomes, that are adjuvanted with innate receptor ligands could form an attractive approach for induction of safe and effective RSV-specific immunity.

Non-replicating RSV particles: virosomes

Virosomes are non-replicating virus particles, consisting of reconstituted viral membranes carrying viral surface glycoproteins [125]. Virosomes are produced from virus by solubilization of viral membranes by detergent Octaethyleneglycol mono (n-dodecyl) ether (C12E8) or 1, 2-dicaproyl-sn-glycero-3-phosphocholine (DCPC) followed by nucleocapsid removal and subsequent reconstitution of the solubilized membranes [126,127]. The advantage of use of DCPC vs C12E8 is that it can be removed through dialysis due to its high critical micelle concentration (cmc) [127]. Previously, this approach has been used for the preparation of RSV virosomes [122]. These particles also provide a platform for incorporation of lipophilic adjuvants, like lipophilic TLR ligands, together with the viral protein antigens [122].

Mucosal administration of vaccine

Mucosal surfaces comprise the largest surface area between the body and the external environment. Most infectious agents like RSV enter the body at mucosal surfaces and therefore, mucosal immune responses at these sites could provide a first line of defense. Mucosal immunization (e.g. intranasal; IN) can provide significant protection against RSV by inducing both systemic and local antibodies, particularly secretory IgA (S-IgA) and can contribute to inhibition of infection by respiratory pathogens [128,129]. Pathogens or any foreign antigen is transported across the epithelial barrier by specialized antigen transporting cells (i.e. M-cells). Immune responses can then be initiated in local lymphoid tissues that are situated in close proximity to the mucosal surface. In the respiratory tract this lymphoid tissue includes the nasal-associated lymphoid tissue (NALT) and the bronchus-associated lymphoid tissue (BALT). Additionally, mucosal DCs aid in transport of antigens to local draining lymph nodes where they activate T and B cells, leading to the initiation of adaptive immune responses including the induction of S-IgA at the mucosal surfaces [124]. The activated T and B cells also acquire mucosal homing properties in the draining lymph nodes from specialized

DCs that migrate from the mucosal tissues to these lymph nodes [130]. Mucosal immunization can induce both humoral and cell-mediated immune protection not only at the mucosal sites but also systemically [131,132]. In addition, mucosal immunization can induce long-term B and T cell memory [133,134]. This route of administration is may, therefore, be quite suitable for use in vaccination strategies.

Mucosal vaccine adjuvants

Induction of immune responses through deposition of non-replicating vaccine antigens to mucosal tissues, like the nasal mucosal tissue, in general needs the use of antigen carrier systems and/or adjuvants [135]. Potent mucosal adjuvants are bacterial derived enterotoxins like cholera toxin (CT) or *E. coli* heat-labile toxin (LT). They bind via their B subunits to gangliosides that are present on the cell membrane of most nucleated cells [136]. These gangliosides are ubiquitously expressed, and so these adjuvants may be associated with unwanted side-effects. For example, IN immunization of a virosomal influenza vaccine supplemented with *E. coli* heat-labile toxin as adjuvant has resulted in facial paralysis (i.e. Bell's palsy syndrome) in a number of vaccinated individuals reviewed in [124]. Another new class of adjuvants suitable as mucosal adjuvants is represented by TLR agonists.

TLRs and NLRs belong to the family of pattern recognition receptors (PRRs) and recognize molecules that are broadly shared by pathogens but distinguishable from the host molecules as described above in detail in (innate immune responses section). Activation of TLR/NLR leads to recruitment of adaptor molecules like MyD88 or TRIF that activate transcription factors like Nuclear Factor-kappa B (NF-κB) or IRF's (Figure 2: TLRs/NLRs signaling pathways). Depending on the type of TLR/NLR ligand used, activation leads for example to the production of pro-inflammatory cytokines, type I IFN's and the expression of co-stimulatory molecules on DCs. In this way, TLR engagement has the ability to direct the adaptive immune response towards a Th1-type reaction [137]. TLR adjuvants constitute a major class of mucosal adjuvants [138]. Some examples of TLR ligands that show promise for use in vaccines, including mucosally delivered RSV vaccines, are illustrated below.

MPLA

Monophosphoryl lipid A (MPLA) is a TLR4 ligand derived from the detoxified form of LPS. This is the first recognized and only TLR ligand molecule approved for use in humans which signals through TLR4 [139]. Currently, two MPLA-adjuvanted vaccines are licensed for human use: a human papillomavirus vaccine, Cervarix [140,141] and Fendrix, a hepatitis B virus (HBV) vaccine for patients with renal insufficiency [142]. A candidate vaccine against herpes simplex virus (HSV) also has been co-formulated with MPLA and has recently been tested in a phase III clinical trial. This formulation incorporates the adjuvant system AS04, which is composed of alum with MPLA [143-145]. It is important to note that MPLA has the capacity to suppress the symptoms of ERD when co-administered with FI-

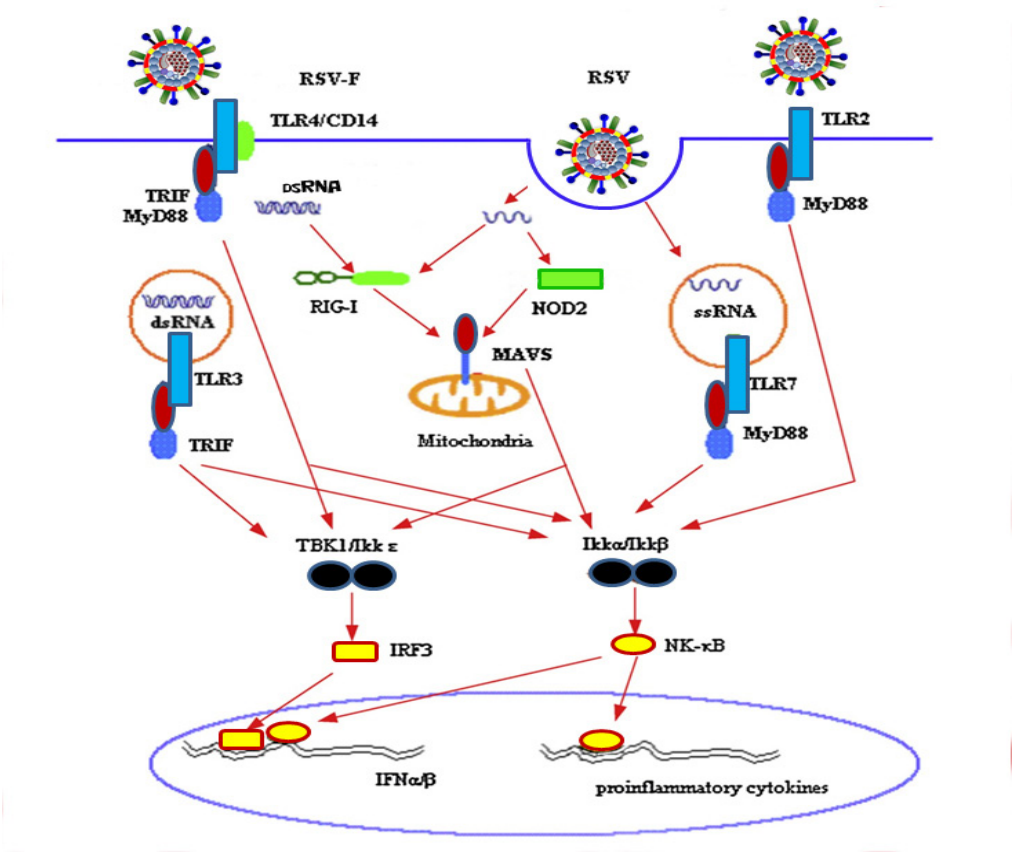

Figure 2: Detection of RSV by TLRs and NLRs. TLR4 is expressed on the surface of immune cells and recognizes RSV F protein. TLR3 is expressed in intracellular endosomes and recognizes dsRNA, that is produced during RSV replication. Both TLR3 and TLR4 bind with TRIF and also activate the kinases TBK1/IKKε and IKKα/IKKβ which result into phosphorylation and translocation of IRF3 and NF-κB to the nucleus. This phenomenon leads to expression of IFN-α/β and pro-inflammatory cytokines. TLR7 recognizes the ssRNA of RSV in the intracellular endosomes. TLR2 and TLR4 on the surface of immune cells and TLR7 inside the cells signal through MyD88-dependent pathway, leading to the translocation of NF-κB. This leads to the release of IFN-α/β and pro-inflammatory cytokines. NOD2 may recognize the ssRNA of RSV and activate the downstream NF-κB and IRF3 pathways by making a complex with the MAVS adaptor that localizes to the mitochondrial membrane (Adapted from [52]).

RSV to cotton rats [146,147], which underlines its potentially beneficial role as an adjuvant in RSV vaccines.

CpG
CpG DNAs are synthetic oligonucleotides that contain unmethylated CpG dinucleotides and are recognized by Toll-Like receptor 9 (TLR9) [148]. TLR9 is expressed intracellularly within the endosomal cell compartment and functions to alert the immune system to viral and bacterial infections by binding to DNA rich in CpG motifs. TLR9 is expressed by different cells of the immune system such as macrophages, B cells, DCs, monocytes and NK cells. CpG DNA has been reported to induce Th1-skewed immune responses and may also act as promising mucosal adjuvant [149,150]. CpG DNA has been found to induce Th1-skewed immune responses in animal studies after mucosal administration together with the vaccine antigens [149,151-153].

Pam_3CSK_4
N-pamitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-(lysyl)3-lysine (Pam_3CSK_4), for example, is a synthetic triacylated lipopeptide with a similar bioactivity as lipopeptides from Gram-negative bacteria [154]. It can be easily incorporated in virosomal membranes upon their reconstitution [122]. It is being recognized through TLR2 at the cell surface [155] and has been shown to possess beneficial adjuvant effects for use, for example, in vaccines [156]. When incorporated in RSV-virosomes, Pam_3CSK_4 enhances RSV-specific serum IgG and Th1 responses upon intramuscular immunization [122].

L18-MDP
L18-MDP is a synthetic derivative of 6-O-stearoyl-N-Acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl-di-peptide; MDP). L18 refers to the stearoyl fatty acid attached to MDP. MDP belongs to another class of innate receptors ligands that activate NOD-like receptors (NLRs). NLRs are located in the cytosol and consist of three domains characterized by an N-terminal effector and a central nucleotide-binding domain (NBD) as well as C-terminal multiple leucine-rich repeats (LRR) [157,158]. NOD1- and NOD2-receptors are known to recognize fragments of bacterial peptidoglycans (PGN), like MDP motifs [159]. (L18-)MDP induces pro-inflammatory cytokines and antibacterial responses through activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways [159]. L18-MDP (a NOD2 ligand) has been found 10 times more efficient than MDP to induce NF-κB activation *in vitro* and also showed the highest activity in mice [160]. Furthermore, L18-MDP has been reported to have mucosal immunoadjuvant properties [161,162].

Scope and outline of this thesis

Mucosal administration represents an attractive potential approach to vaccination against RSV. In this strategy, innate receptor ligands, like TLR or NLR ligands, could be employed since these have been shown to possess mucosal immunoadjuvant activity. In this thesis, the use of TLR/NLR ligands in non-replicating RSV vaccines, administered intranasally (IN) to mice and cotton rats, is explored.

Chapter 2 describes an exploratory study investigating the capacity of a widely used TLR ligand (CpG ODN) alone, or supplemented with a NOD2 ligand (L18-MDP), to enhance systemic and mucosal antibody responses when simply mixed with a model antigen (inactivated RSV particles) upon IN administration to mice.

Subsequently, in order to formulate an RSV vaccine with built-in adjuvant activity, RSV virosomes were produced with lipophilic TLR ligands, such as Pam_3CSK_4 and/or L18-MDP, incorporated in the virosomal membranes **(Chapter 3)**. Their immunopotentiating capacity was tested *in vitro* and *in vivo* in mice **(Chapter 3)**. Also, the protective capacity of this vaccine was assessed after IN administration to cotton rats, an animal model that is more permissive for RSV infection **(Chapter 4)**.

An attractive TLR adjuvant for use in a virosomal RSV vaccine is MPLA, not only because it is lipophilic allowing its incorporation in virosomal membranes, but also because it is already used in registered human vaccines. Its use as an adjuvant in an IN virosomal RSV vaccine was therefore explored in **Chapter 5.**

Finally, since the elderly represent an important target population for a non-replicating RSV vaccine, the capacity of RSV virosomes with incorporated MPLA to induce immunity in an aged immune system was investigated. To this end, the vaccine was tested in aged cotton rats with or without pre-existing immunity to RSV **(Chapter 6)**.

In **Chapter 7**, the results are summarized and discussed and future perspectives for RSV vaccine development are presented.

References:

1. Dowell SF, Anderson LJ, Gary HE,Jr., Erdman DD, Plouffe JF, File TM,Jr. et al. Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults. J Infect Dis 1996;174(3):456-62.
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(17):1749-59.
3. Lamb RA, Jardetzky TS. Structural basis of viral invasion: lessons from paramyxovirus F. Curr Opin Struct Biol 2007;17(4):427-36.
4. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y et al. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis 1985;151(4):626-33.
5. Hacking D, Hull J. Respiratory syncytial virus--viral biology and the host response. J Infect 2002;45(1):18-24.
6. Spann KM, Tran KC, Collins PL. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J Virol 2005;79(9):5353-62.
7. Feldman SA, Audet S, Beeler JA. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. J Virol 2000;74(14):6442-7.
8. Feldman SA, Crim RL, Audet SA, Beeler JA. Human respiratory syncytial virus surface glycoproteins F, G and SH form an oligomeric complex. Arch Virol 2001;146(12):2369-83.
9. Gonzalez PA, Bueno SM, Carreno LJ, Riedel CA, Kalergis AM. Respiratory syncytial virus infection and immunity. Rev Med Virol 2012;22(4):230-44.
10. Cheng X, Park H, Zhou H, Jin H. Overexpression of the M2-2 protein of respiratory syncytial virus inhibits viral replication. J Virol 2005;79(22):13943-52.
11. Fearns R, Collins PL. Role of the M2-1 transcription antitermination protein of respiratory syncytial virus in sequential transcription. J Virol 1999;73(7):5852-64.
12. Spence L, Barratt N. Respiratory syncytial virus associated with acute respiratory infections in Trinidadian patients. Am J Epidemiol 1968;88(2):257-66.
13. Huq F, Rahman M, Nahar N, Alam A, Haque M, Sack DA et al. Acute lower respiratory tract infection due to virus among hospitalized children in Dhaka, Bangladesh. Rev Infect Dis 1990;12 Suppl 8:S982-7.
14. Vardas E, Blaauw D, McAnerney J. The epidemiology of respiratory syncytial virus (RSV) infections in South African children. S Afr Med J 1999;89(10):1079-84.
15. Byrd LG, Prince GA. Animal models of respiratory syncytial virus infection. Clin Infect Dis 1997;25(6):1363-8.
16. Hall CB, Douglas RG,Jr. Modes of transmission of respiratory syncytial virus. J Pediatr 1981;99(1):100-3.
17. Henderson FW, Collier AM, Clyde WA,Jr, Denny FW. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N Engl J Med 1979;300(10):530-4.
18. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 1991;163(4):693-8.
19. Reed G, Jewett PH, Thompson J, Tollefson S, Wright PF. Epidemiology and clinical impact of parainfluenza virus infections in otherwise healthy infants and young children < 5 years old. J Infect Dis 1997;175(4):807-13.
20. Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999;282(15):1440-6.
21. Tissing WJ, van Steensel-Moll HA, Offringa M. Risk factors for mechanical ventilation in respiratory syncytial virus infection. Eur J Pediatr 1993;152(2):125-7.
22. Green M, Brayer AF, Schenkman KA, Wald ER. Duration of hospitalization in previously well infants with respiratory syncytial virus infection. Pediatr Infect Dis J 1989;8(9):601-5.
23. Navas L, Wang E, de Carvalho V, Robinson J. Improved outcome of respiratory syncytial

virus infection in a high-risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J Pediatr 1992;121(3):348-54.
24. Groothuis JR, Gutierrez KM, Lauer BA. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 1988;82(2):199-203.
25. MacDonald NE, Hall CB, Suffin SC, Alexson C, Harris PJ, Manning JA. Respiratory syncytial viral infection in infants with congenital heart disease. N Engl J Med 1982;307(7):397-400.
26. Hall CB, Powell KR, MacDonald NE, Gala CL, Menegus ME, Suffin SC et al. Respiratory syncytial viral infection in children with compromised immune function. N Engl J Med 1986;315(2):77-81.
27. Welliver RC. Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. J Pediatr 2003;143(5 Suppl):S112-7.
28. Hall CB, Long CE, Schnabel KC. Respiratory syncytial virus infections in previously healthy working adults. Clin Infect Dis 2001;33(6):792-6.
29. Han LL, Alexander JP, Anderson LJ. Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J Infect Dis 1999;179(1):25-30.
30. Falsey AR, Walsh EE. Respiratory syncytial virus infection in adults. Clin Microbiol Rev 2000;13(3):371-84.
31. Hall WJ, Hall CB, Speers DM. Respiratory syncytial virus infection in adults: clinical, virologic, and serial pulmonary function studies. Ann Intern Med 1978;88(2):203-5.
32. Miyairi I, DeVincenzo JP. Human genetic factors and respiratory syncytial virus disease severity. Clin Microbiol Rev 2008;21(4):686-703.
33. Culley FJ, Pennycook AM, Tregoning JS, Hussell T, Openshaw PJ. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. J Virol 2006;80(0022-538; 0022-538; 9):4521-7.
34. Garofalo RP, Patti J, Hintz KA, Hill V, Ogra PL, Welliver RC. Macrophage inflammatory protein-1alpha (not T helper type 2 cytokines) is associated with severe forms of respiratory syncytial virus bronchiolitis. J Infect Dis 2001;184(4):393-9.
35. Parrott RH, Kim HW, Arrobio JO, Hodes DS, Murphy BR, Brandt CD et al. Epidemiology of respiratory syncytial virus infection in Washington, D.C. II. Infection and disease with respect to age, immunologic status, race and sex. Am J Epidemiol 1973;98(4):289-300.
36. Brandenburg AH, Jeannet PY, Steensel-Moll HA, Ott A, Rothbarth PH, Wunderli W et al. Local variability in respiratory syncytial virus disease severity. Arch Dis Child 1997;77(5):410-4.
37. Singleton RJ, Petersen KM, Berner JE, Schulte E, Chiu K, Lilly CM et al. Hospitalizations for respiratory syncytial virus infection in Alaska Native children. Pediatr Infect Dis J 1995;14(1):26-30.
38. McConnochie KM, Roghmann KJ. Breast feeding and maternal smoking as predictors of wheezing in children age 6 to 10 years. Pediatr Pulmonol 1986;2(5):260-8.
39. Wright AL, Holberg C, Martinez FD, Taussig LM. Relationship of parental smoking to wheezing and nonwheezing lower respiratory tract illnesses in infancy. Group Health Medical Associates. J Pediatr 1991;118(2):207-14.
40. Groothuis JR, Gutierrez KM, Lauer BA. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics 1988;82(2):199-203.
41. Jung JW. Respiratory syncytial virus infection in children with congenital heart disease: global data and interim results of Korean RSV-CHD survey. Korean J Pediatr 2011;54(5):192-6.
42. Walsh EE, McConnochie KM, Long CE, Hall CB. Severity of respiratory syncytial virus infection is related to virus strain. J Infect Dis 1997;175(4):814-20.
43. Hornsleth A, Klug B, Nir M, Johansen J, Hansen KS, Christensen LS et al. Severity of respiratory syncytial virus disease related to type and genotype of virus and to cytokine values in nasopharyngeal secretions. Pediatr Infect Dis J 1998;17(12):1114-21.
44. Devincenzo JP. Natural infection of infants with respiratory syncytial virus subgroups A and B: a study of frequency, disease severity, and viral load. Pediatr Res 2004;56(6):914-7.
45. DeVincenzo JP, El Saleeby CM, Bush AJ. Respiratory syncytial virus load predicts disease

severity in previously healthy infants. J Infect Dis 2005;191(11):1861-8.
46. Van Drunen Littel-van den Hurk,S., Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. Rev Med Virol 2007;17(1):5-34.
47. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89(4):422-34.
48. 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(4):435-48.
49. 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(4):449-63.
50. Chanock R, Finberg L. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). II. Epidemiologic aspects of infection in infants and young children. Am J Hyg 1957;66(3):291-300.
51. Sung SS, Fu SM, Rose CE,Jr, Gaskin F, Ju ST, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J Immunol 2006;176(4):2161-72.
52. Zeng R, Cui Y, Hai Y, Liu Y. Pattern recognition receptors for respiratory syncytial virus infection and design of vaccines. Virus Res 2012;167(2):138-45.
53. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 2000;1(5):398-401.
54. Suzuki T, Chow CW, Downey GP. Role of innate immune cells and their products in lung immunopathology. Int J Biochem Cell Biol 2008;40(6-7):1348-61.
55. Haeberle HA, Takizawa R, Casola A, Brasier AR, Dieterich HJ, Van Rooijen N et al. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. J Infect Dis 2002;186(9):1199-206.
56. Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G et al. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J Biol Chem 2003;278(52):53035-44.
57. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH et al. Activation of innate immune antiviral responses by Nod2. Nat Immunol 2009;10(10):1073-80.
58. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245-52.
59. Fishaut M, Tubergen D, McIntosh K. Cellular response to respiratory viruses with particular reference to children with disorders of cell-mediated immunity. J Pediatr 1980;96(2):179-86.
60. Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K et al. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. J Infect Dis 2007;195(8):1126-36.
61. Lydyard MP, Immunology. 6th edition. 2001.
62. Becker Y. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review. Virus Genes 2006;33(2):235-52.
63. Bendelja K, Gagro A, Bace A, Lokar-Kolbas R, Krsulovic-Hresic V, Drazenovic V et al. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. Clin Exp Immunol 2000;121(2):332-8.
64. Brandenburg AH, Kleinjan A, van Het Land B, Moll HA, Timmerman HH, de Swart RL et al. Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. J Med Virol 2000;62(2):267-77.
65. Van Schaik SM, Tristram DA, Nagpal IS, Hintz KM, Welliver RC,2nd, Welliver RC. Increased production of IFN-gamma and cysteinyl leukotrienes in virus-induced wheezing. J Allergy

Clin Immunol 1999;103(4):630-6.
66. Kristjansson S, Bjarnarson SP, Wennergren G, Palsdottir AH, Arnadottir T, Haraldsson A et al. Respiratory syncytial virus and other respiratory viruses during the first 3 months of life promote a local TH2-like response. J Allergy Clin Immunol 2005;116(4):805-11.
67. 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(0022-538; 0022-538; 5):2852-60.
68. Hancock GE, Speelman DJ, Heers K, Bortell E, Smith J, Cosco C. Generation of atypical pulmonary inflammatory responses in BALB/c mice after immunization with the native attachment (G) glycoprotein of respiratory syncytial virus. J Virol 1996;70(0022-538; 0022-538; 11):7783-91.
69. Jackson M, Scott R. Different patterns of cytokine induction in cultures of respiratory syncytial (RS) virus-specific human TH cell lines following stimulation with RS virus and RS virus proteins. J Med Virol 1996;49(3):161-9.
70. Johnson TR, Johnson JE, Roberts SR, Wertz GW, Parker RA, Graham BS. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. J Virol 1998;72(4):2871-80.
71. Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HC,III, Sotnikov AV et al. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J Virol 1992;66(0022-538; 0022-538; 12):7444-51.
72. Connors M, Giese NA, Kulkarni AB, Firestone CY, Morse HC,III, Murphy BR. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. J Virol 1994;68(0022-538; 0022-538; 8):5321-5.
73. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol Rev 2011;239(1):149-66.
74. Nicholas JA, Rubino KL, Levely ME, Adams EG, Collins PL. Cytolytic T-lymphocyte responses to respiratory syncytial virus: effector cell phenotype and target proteins. J Virol 1990;64(9):4232-41.
75. Mbawuike IN, Wells J, Byrd R, Cron SG, Glezen WP, Piedra PA. HLA-restricted CD8+ cytotoxic T lymphocyte, interferon-gamma, and interleukin-4 responses to respiratory syncytial virus infection in infants and children. J Infect Dis 2001;183(5):687-96.
76. Heidema J, Lukens MV, van Maren WW, van Dijk ME, Otten HG, van Vught AJ et al. CD8+ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. J Immunol 2007;179(12):8410-7.
77. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991;88(3):1026-33.
78. Alwan WH, Record FM, Openshaw PJ. CD4+ T cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T cells. Clin Exp Immunol 1992;88(3):527-36.
79. Bangham CR, Openshaw PJ, Ball LA, King AM, Wertz GW, Askonas BA. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoprotein (G), expressed by vaccinia virus recombinants. J Immunol 1986;137(12):3973-7.
80. Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J Exp Med 1988;168(3):1163-8.
81. Welliver RC, Kaul TN, Putnam TI, Sun M, Riddlesberger K, Ogra PL. The antibody response to primary and secondary infection with respiratory syncytial virus: kinetics of class-specific responses. J Pediatr 1980;96(5):808-13.
82. Prince GA, Hemming VG, Horswood RL, Baron PA, Chanock RM. Effectiveness of topically administered neutralizing antibodies in experimental immunotherapy of respiratory syncytial virus infection in cotton rats. J Virol 1987;61(6):1851-4.

83. Siber GR, Leombruno D, Leszczynski J, McIver J, Bodkin D, Gonin R et al. Comparison of antibody concentrations and protective activity of respiratory syncytial virus immune globulin and conventional immune globulin. J Infect Dis 1994;169(6):1368-73.
84. Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. J Infect Dis 2004;190(2):373-8.
85. Walsh EE, Peterson DR, Falsey AR. Risk factors for severe respiratory syncytial virus infection in elderly persons. J Infect Dis 2004;189(2):233-8.
86. Falsey AR, Walsh EE. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. J Infect Dis 1998;177(2):463-6.
87. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. Mod Pathol 2007;20(1):108-19.
88. Singleton R, Etchart N, Hou S, Hyland L. Inability to evoke a long-lasting protective immune response to respiratory syncytial virus infection in mice correlates with ineffective nasal antibody responses. J Virol 2003;77(0022-538; 0022-538; 21):11303-11.
89. Valosky J, Hishiki H, Zaoutis TE, Coffin SE. Induction of mucosal B-cell memory by intranasal immunization of mice with respiratory syncytial virus. Clin Diagn Lab Immunol 2005;12(1071-412; 1071-412; 1):171-9.
90. Weltzin R, Hsu SA, Mittler ES, Georgakopoulos K, Monath TP. Intranasal monoclonal immunoglobulin A against respiratory syncytial virus protects against upper and lower respiratory tract infections in mice. Antimicrob Agents Chemother 1994;38(12):2785-91.
91. Fisher RG, Crowe JE,Jr., Johnson TR, Tang YW, Graham BS. Passive IgA monoclonal antibody is no more effective than IgG at protecting mice from mucosal challenge with respiratory syncytial virus. J Infect Dis 1999;180(4):1324-7.
92. Wang EE, Tang NK. Immunoglobulin for preventing respiratory syncytial virus infection. Cochrane Database Syst Rev 2000;(2)(2):CD001725.
93. Nokes JD, Cane PA. New strategies for control of respiratory syncytial virus infection. Curr Opin Infect Dis 2008;21(6):639-43.
94. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. The IMpact-RSV Study Group. Pediatrics 1998;102(3 Pt 1):531-7.
95. Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK et al. Development of motavizumab, an ultra-potent antibody for the prevention of respiratory syncytial virus infection in the upper and lower respiratory tract. J Mol Biol 2007;368(3):652-65.
96. Ventre K, Randolph AG. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. Cochrane Database Syst Rev 2007;(1)(1):CD000181.
97. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med 2005;11(1):50-5.
98. Zhang W, Yang H, Kong X, Mohapatra S, San Juan-Vergara H, Hellermann G et al. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. Nat Med 2005;11(1):56-62.
99. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969;89(4):405-21.
100. 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(Pt 12):2881-8.
101. Prince GA, Jenson AB, Hemming VG, Murphy BR, Walsh EE, Horswood RL et al. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactiva ted virus. J Virol 1986;57(3):721-8.
102. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP et al. Lack of

antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15(1546-170; 1):34-41.

103. Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat Med 2006;12(8):905-7.
104. Neuzil KM, Johnson JE, Tang YW, Prieels JP, Slaoui M, Gar N et al. Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine. Vaccine 1997;15(5):525-32.
105. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 2008;205(4):869-82.
106. Antonis AF, Schrijver RS, Daus F, Steverink PJ, Stockhofe N, Hensen EJ et al. Vaccine-induced immunopathology during bovine respiratory syncytial virus infection: exploring the parameters of pathogenesis. J Virol 2003;77(22):12067-73.
107. Polack FP, Karron RA. The future of respiratory syncytial virus vaccine development. Pediatr Infect Dis J 2004;23(1 Suppl):S65-73.
108. Wright PF, Karron RA, Belshe RB, Thompson J, Crowe JE,Jr, Boyce TG et al. Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. J Infect Dis 2000;182(5):1331-42.
109. Karron RA, Wright PF, Belshe RB, Thumar B, Casey R, Newman F et al. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J Infect Dis 2005;191(7):1093-104.
110. Piedra PA, Cron SG, Jewell A, Hamblett N, McBride R, Palacio MA et al. Immunogenicity of a new purified fusion protein vaccine to respiratory syncytial virus: a multi-center trial in children with cystic fibrosis. Vaccine 2003;21(19-20):2448-60.
111. Munoz FM, Piedra PA, Glezen WP. Safety and immunogenicity of respiratory syncytial virus purified fusion protein-2 vaccine in pregnant women. Vaccine 2003;21(24):3465-7.
112. Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in ambulatory adults over age 60. Vaccine 1996;14(13):1214-8.
113. Langley JM, Sales V, McGeer A, Guasparini R, Predy G, Meekison W et al. A dose-ranging study of a subunit Respiratory Syncytial Virus subtype A vaccine with and without aluminum phosphate adjuvantation in adults > or =65 years of age. Vaccine 2009;27(42):5913-9.
114. Falsey AR, Walsh EE, Capellan J, Gravenstein S, Zambon M, Yau E et al. Comparison of the safety and immunogenicity of 2 respiratory syncytial virus (rsv) vaccines--nonadjuvanted vaccine or vaccine adjuvanted with alum--given concomitantly with influenza vaccine to high-risk elderly individuals. J Infect Dis 2008;198(9):1317-26.
115. Tang RS, MacPhail M, Schickli JH, Kaur J, Robinson CL, Lawlor HA et al. Parainfluenza virus type 3 expressing the native or soluble fusion (F) Protein of Respiratory Syncytial Virus (RSV) confers protection from RSV infection in African green monkeys. J Virol 2004;78(20):11198-207.
116. Gomez M, Mufson MA, Dubovsky F, Knightly C, Zeng W, Losonsky G. Phase-I study MEDI-534, of a live, attenuated intranasal vaccine against respiratory syncytial virus and parainfluenza-3 virus in seropositive children. Pediatr Infect Dis J 2009;28(7):655-8.
117. Bernstein DI, Malkin E, Abughali N, Falloon J, Yi T, Dubovsky F et al. Phase 1 study of the safety and immunogenicity of a live, attenuated respiratory syncytial virus and parainfluenza virus type 3 vaccine in seronegative children. Pediatr Infect Dis J 2012;31(2):109-14.
118. McGinnes LW, Gravel KA, Finberg RW, Kurt-Jones EA, Massare MJ, Smith G et al. Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. J Virol 2011;85(1):366-77.
119. Schmidt AC. Progress in respiratory virus vaccine development. Semin Respir Crit Care Med 2011;32(4):527-40.
120. Mok H, Lee S, Utley TJ, Shepherd BE, Polosukhin VV, Collier ML et al. Venezuelan equine encephalitis virus replicon particles encoding respiratory syncytial virus surface

glycoproteins induce protective mucosal responses in mice and cotton rats. J Virol 2007;81(24):13710-22.
121. 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(8):1595-7.
122. Stegmann T, Kamphuis T, Meijerhof T, Goud E, de HA, Wilschut J. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. Vaccine 2010;28(0264-410; 34):5543-50.
123. Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol 2007;5(7):505-17.
124. Lawson LB, Norton EB, Clements JD. Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. Curr Opin Immunol 2011;23(3):414-20.
125. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM et al. The virosome concept for influenza vaccines. Vaccine 2005;23 Suppl 1:S26-38.
126. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. EMBO J 1987;6(9):2651-9.
127. De Jonge J, Schoen P, ter Veer W, Stegmann T, Wilschut J, Huckriede A. Use of a dialyzable short-chain phospholipid for efficient solubilization and reconstitution of influenza virus envelopes. Biochim Biophys Acta 2006;1758(4):527-36.
128. Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 2001;14(2):430-45.
129. Baca-Estrada ME, Foldvari M, Babiuk SL, Babiuk LA. Vaccine delivery: lipid-based delivery systems. J Biotechnol 2000;83(1-2):91-104.
130. Mora JR, von Andrian UH. Role of retinoic acid in the imprinting of gut-homing IgA-secreting cells. Semin Immunol 2009;21(1):28-35.
131. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;11(4 Suppl):S45-53.
132. Brandtzaeg P. Function of mucosa-associated lymphoid tissue in antibody formation. Immunol Invest 2010;39(4-5):303-55.
133. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine 2007;25(30):5467-84.
134. Sheridan BS, Lefrancois L. Regional and mucosal memory T cells. Nat Immunol 2011;12(6):485-91.
135. Slutter B, Hagenaars N, Jiskoot W. Rational design of nasal vaccines. J Drug Target 2008;16(1):1-17.
136. Connell TD. Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins. Expert Rev Vaccines 2007;6(5):821-34.
137. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2001;2(10):947-50.
138. Harandi AM, Medaglini D. Mucosal adjuvants. Curr HIV Res 2010;8(4):330-5.
139. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282(5396):2085-8.
140. Descamps D, Hardt K, Spiessens B, Izurieta P, Verstraeten T, Breuer T et al. Safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine for cervical cancer prevention: a pooled analysis of 11 clinical trials. Hum Vaccin 2009;5(5):332-40.
141. Verstraeten T. Rebuttal Letter to the Letter to the Editor to "Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines" by T. Verstraeten et al. Vaccine 2009;27(19):2530.
142. Beran J. Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients. Expert Opin Biol Ther 2008;8(2):235-47.
143. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H et al. AS04, 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(10):6186-97.
144. Garcon N, Segal L, Tavares F, Van Mechelen M. The safety evaluation of adjuvants during

vaccine development: the AS04 experience. Vaccine 2011;29(27):4453-9.
145. Garcon 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(5):667-77.
146. Boukhvalova MS, Prince GA, Soroush L, Harrigan DC, Vogel SN, Blanco JC. The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. Vaccine 2006;24(0264-410; 0264-410; 23):5027-35.
147. Prince GA, Denamur F, Deschamps M, Garcon N, Prieels JP, Slaoui M et al. Monophosphoryl lipid A adjuvant reverses a principal histologic parameter of formalin-inactivated respiratory syncytial virus vaccine-induced disease. Vaccine 2001;19(15-16):2048-54.
148. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408(6813):740-5.
149. Mapletoft JW, Latimer L, Babiuk LA, van Drunen Littel-van dH. Intranasal immunization of mice with a bovine respiratory syncytial virus vaccine induces superior immunity and protection compared to those by subcutaneous delivery or combinations of intranasal and subcutaneous prime-boost strategies. Clin Vaccine Immunol 2010;17(1556-679; 1556-679; 1):23-35.
150. McCluskie MJ, Weeratna RD, Davis HL. Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. Mol Med 2000;6(10):867-77.
151. Huang CF, Wu TC, Chu YH, Hwang KS, Wang CC, Peng HJ. Effect of neonatal sublingual vaccination with native or denatured ovalbumin and adjuvant CpG or cholera toxin on systemic and mucosal immunity in mice. Scand J Immunol 2008;68(5):502-10.
152. Pesce I, Monaci E, Muzzi A, Tritto E, Tavarini S, Nuti S et al. Intranasal administration of CpG induces a rapid and transient cytokine response followed by dendritic and natural killer cell activation and recruitment in the mouse lung. J Innate Immun 2010;2(2):144-59.
153. Prince GA, Mond JJ, Porter DD, Yim KC, Lan SJ, Klinman DM. Immunoprotective activity and safety of a respiratory syncytial virus vaccine: mucosal delivery of fusion glycoprotein with a CpG oligodeoxynucleotide adjuvant. J Virol 2003;77(24):13156-60.
154. Kovacs-Simon A, Titball RW, Michell SL. Lipoproteins of bacterial pathogens. Infect Immun 2011;79(2):548-61.
155. Melchers F, Braun V, Galanos C. The lipoprotein of the outer membrane of Escherichia coli: a B-lymphocyte mitogen. J Exp Med 1975;142(2):473-82.
156. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science 1999;285(5428):736-9.
157. Martinon F, Tschopp J. NLRs join TLRs as innate sensors of pathogens. Trends Immunol 2005;26(8):447-54.
158. Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nat Rev Immunol 2003;3(5):371-82.
159. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006;6(1):9-20.
160. Matsumoto K, Ogawa H, Kusama T, Nagase O, Sawaki N, Inage M et al. Stimulation of nonspecific resistance to infection induced by 6-O-acyl muramyl dipeptide analogs in mice. Infect Immun 1981;32(2):748-58.
161. Fukushima A, Yoo YC, Yoshimatsu K, Matsuzawa K, Tamura M, Tono-oka S et al. Effect of MDP-Lys(L18) as a mucosal immunoadjuvant on protection of mucosal infections by Sendai virus and rotavirus. Vaccine 1996;14(0264-410; 0264-410; 6):485-91.
162. Ogawa T, Shimauchi H, Hamada S. Mucosal and systemic immune responses in BALB/c mice to Bacteroides gingivalis fimbriae administered orally. Infect Immun 1989;57(11):3466-71.

Chapter 2

Induction of mucosal and systemic immunity against Respiratory Syncytial Virus by inactivated virus supplemented with TLR9 and NOD2 ligands

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Abstract

Respiratory syncytial virus (RSV) infection is the most important viral cause of severe respiratory disease in infants and children worldwide and also forms a serious threat in the elderly. The development of RSV vaccine, however, has been hampered by the disastrous outcome of an earlier trial using an inactivated and parenterally administered RSV vaccine which did not confer protection but rather primed for enhanced disease upon natural infection. Mucosal administration does not seem to prime for enhanced disease, but non-replicating RSV antigen does not induce a strong mucosal immune response. We therefore investigated if mucosal immunization with inactivated RSV supplemented with innate receptor ligands, TLR9 (CpG ODN) and NOD2 (L18-MDP) through the upper or total respiratory tract is an effective and safe approach to induce RSV-specific immunity. Our data show that beta-propiolactone (BPL) inactivated RSV (BPL-RSV) supplemented with CpG ODN and L18-MDP potentiates activation of antigen-presenting cells (APC) *in vitro*, as demonstrated by NF-κB induction in a model APC cell line. *In vivo*, BPL-RSV supplemented with CpG ODN/L18-MDP ligands induces local IgA responses and augments Th1-signature IgG2a subtype responses after total respiratory tract (TRT), but less efficient after upper respiratory tract (intranasal,IN) immunization. Addition of TLR9/NOD2 ligands to the inactivated RSV also promoted affinity maturation of RSV-specific IgG antibodies and shifted T cell responses from mainly IL-5-secreting cells to predominantly IFN-γ-producing cells, indicating a Th1-skewed response. This effect was seen for both IN and TRT immunization. Finally, BPL-RSV supplemented with TLR9/NOD2 ligands significantly improved the protection efficacy against a challenge with infectious virus, without stimulating enhanced disease as evidenced by lack of eotaxin mRNA expression and eosinophil infiltration in the lung. We conclude that mucosal immunization with inactivated RSV antigen supplemented with TLR9/NOD2 ligands is a promising approach to induce effective RSV-specific immunity without priming for enhanced disease.

1. Introduction

Respiratory syncytial virus (RSV) is the main cause of lower respiratory tract infections among infants and young children. Since natural infection with RSV results in incomplete immunity, reinfection occurs frequently throughout life. For this reason, RSV infection also represents a serious threat in chronically ill adults and the elderly [1]. Recent studies have demonstrated that RSV makes a major contribution to mortality in the elderly, as does influenza [2]. However, despite the impact of RSV infection, a safe and efficacious vaccine is not available.

RSV vaccine development has been hampered by the disastrous outcome of a clinical trial with formalin-inactivated RSV (FI-RSV) vaccine in infants in the 1960s. It appeared that vaccination with FI-RSV did not confer protection but rather primed for enhanced disease upon exposure to live RSV, leading to the death of two children [3]. The enhanced disease was linked to severe immunopathology, with hallmarks such as peribronchiolar inflammation, airway obstruction, abundant neutrophils and eosinophils and local immune complex deposition [4]. Later studies in mice showed that FI-RSV-primed cellular immune responses are strongly skewed towards a Th2-type upon RSV challenge [5]. Such responses were accompanied by persistent chemokine responses, e.g. CCL11 (eotaxin), with influx of eosinophils [6,7]. Not only FI-RSV vaccine, but also other types of non-replicating RSV vaccines, including whole virus vaccines inactivated by other treatments than formaldehyde (e.g. UV-treated RSV particles), can prime for enhanced disease in animal models. This property has been particularly attributed to a lack of antibody affinity maturation induced by these types of vaccines, leading to production of poorly neutralizing antibodies, just as in the case of FI-RSV vaccine [8].

Mucosal immunization routes may reduce the risk of enhanced disease and increase vaccine efficacy. Unlike parenteral immunization, mucosal immunization does not readily prime for enhanced disease in animal models [9]. Moreover, mucosal immunization might further promote vaccine efficacy by induction of mucosal IgA, which can act as a first-line immune defense against the virus. Several lines of evidence suggest that RSV-specific IgA antibodies are secreted rapidly in the upper airways following a primary RSV infection in mice [10]. These RSV-specific IgA antibodies have been shown to confer protection against RSV in mice, although in humans their role in protection has remained unclear [11-14]. Optimal induction of such mucosal immune responses, including mucosal IgA in the respiratory tract, requires local deposition of antigen [15]. It is unknown however which part of the respiratory tract, e.g. the upper respiratory tract or lower respiratory tract, should be targeted for induction of sufficiently protective and safe RSV-specific immunity. Thus, mucosal immunization through the respiratory tract could enhance safety and efficacy of immunization against RSV, but needs further exploration with respect to the site of antigen deposition for induction of maximum immune responses.

In addition to use of alternative vaccination routes, i.e. mucosal vaccination, supplementation of the RSV antigen with adjuvants might prevent priming for enhanced disease. For example, addition of innate receptor ligands, such as TLR ligands, to non-replicating RSV particles may lead to Th1-skewed responses, which prevent priming for enhanced disease [16-18]. In a previous study, we showed that incorporation of the TLR2 ligand (Pam_3CSK_4) in a virosomal RSV vaccine skewed responses to a protective and safe Th1-type response [18]. Importantly, addition of TLR ligands to inactivated RSV effectively induces antibody affinity maturation, leading to strongly neutralizing antibodies without priming for enhanced disease [8]. Interestingly, TLR ligand-induced Th1 responses have been reported to be synergistically enhanced by NOD2 ligands, another class of innate receptor ligands [19]. Importantly, both TLR and NOD2 ligands may act as mucosal adjuvants also [20,21]. Thus, mucosal immunization with non-replicating RSV antigen supplemented with TLR9/NOD2 ligands, may well represent a safe approach to induce RSV-specific immunity. It may also be highly effective through induction of mucosal RSV-specific IgA antibodies [22-24].

In the present study, we investigated whether administration of beta-propiolactone (BPL)-inactivated whole RSV (BPL-RSV) supplemented with CpG oligodeoxynucleotides (CpG ODN) and L18-muramyl dipeptide (L18-MDP) to the upper or total respiratory tract of mice would form an effective and safe approach to induce RSV-specific immunity.

2. Materials and Methods

2.1 Preparation of inactivated RSV

Purified RSV (A2 strain; American Type Culture Collection, ATCC) was kindly donated by Mymetics BV, Leiden, The Netherlands. BPL-inactivated RSV was produced by inactivating the virus with 0.025% BPL (Acros Organics, Amsterdam, The Netherlands) for 16 h at 4°C under gentle shaking followed by 2 h of incubation at 37°C to inactivate the BPL. Then, inactivated virus was dialysed against HNE buffer containing 5 mM Hepes, 145 mM NaCl, 1 mM EDTA (pH 7.6). Formalin-inactivated RSV (FI-RSV) vaccine absorbed to aluminium hydroxide was prepared as described before [18].

2.2 Macrophage NF-κB activation by vaccine formulations in vitro

RAW-Blue™ cells (mouse macrophage reporter cell line; InvivoGen, Toulouse, France) were used to measure vaccine/innate receptor ligand-induced NF-κB activation. RAW-blue cells express all TLR (with the exception of TLR5) as well as RIG-I, MDA5, NOD1 and NOD2. Cells were placed in 96-wells plates at $1x10^5$ cells/well. Next, cells were co-incubated with different concentrations of CpG ODN (ODN1826, TCCATGACGTTCCTGACGTT; Eurogentec, Maastricht, The Netherlands) and L18-MDP (6-*o*-stearoyl-N-Acetyl-muramyl-L-alanyl-D-isoglutamine; InvivoGen, Toulouse, France) with or without BPL-RSV

(10 µg/mL) overnight at 37°C, 5% CO_2 atmosphere. NF-κB activation was determined by a colorimetric assay, measuring substrate cleavage by alkaline phosphatase in the supernatant. This enzyme is produced and secreted by RAW-Blue cells after induction of the NF-κB-responsive reporter gene in these cells.

2.3 Immunization schedule and RSV challenge

Animal experiments were evaluated and approved by the Committee for Animal Experimentation of the University Medical Center Groningen, University of Groningen, The Netherlands, according to the guidelines provided by the Dutch Animal Protection Act. Female specified-pathogen-free BALB/c OlaHsd mice (6-8 weeks old) purchased from Harlan, Zeist, The Netherlands, were used for all immunization experiments. Mice were immunized either with BPL-RSV (5 µg) alone or mixed with innate receptor ligands, i.e. 4 µg CpG ODN and 0.1 µg L18-MDP. Mice (6 mice per group) were immunized on days 0 and 21, under isoflurane anesthesia by intranasal inoculation of 10 µL (intranasal immunization confining the inoculum to the upper respiratory tract, i.e. nasal cavity, only; IN;[25]) or 50 µL (total respiratory tract immunization; TRT;[25]). In experiments investigating lung cytokine levels and the influx of eosinophils in the lungs, a control group was immunized with formalin-inactivated RSV vaccine by intramuscular injection of 25 µL of FI-RSV absorbed to aluminium hydroxide (see above). All mice were challenged with live virus by administration of 5 x 10 µL (2 x 10^6 $TCID_{50}$) of virus in the nose under isoflurane anesthesia.

2.4 Collection of blood samples and mucosal washes

Blood samples were drawn twice during the experiment: on day 28 before challenge by orbital puncture and on day 32 by heart puncture. Sera were obtained after centrifugation of coagulated blood at 12,000 rpm for 10 min, and samples were stored at −20°C until further analysis. Bronchoalveolar lavages (BAL) and nasal washes were performed as previously described [26]. Briefly, lung lavages were performed by gentle injection of 1 mL PBS into the lungs with a syringe connected to the trachea, followed by subsequent aspiration of 1 mL of the wash fluid. Nasal washes were done by injection of 0.5 mL PBS retrograde via the trachea into the naso-pharynx and the lavage fluid was collected at the nostrils. Cellular components in the washes were removed by low-speed centrifugation and used for cytospots (see below) or discarded. The supernatants were stored at −20°C until further analysis.

2.5 Antibody titer determination through ELISA

The antibody response to RSV was determined using enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) were coated with BPL-RSV at 0.5 µg protein per well in coating buffer (0.05M carbonate–bicarbonate, pH 9.6–9.8) overnight at

37°C. Plates were washed three times with coating buffer and blocked with a 2.5% solution of Protifar Plus (Nutricia, Zoetermeer, The Netherlands) in coating buffer for 45 min at 37°C, then washed twice with coating buffer and three times with PBS Tween (PBST), containing 0.05% Tween 20 (Merck, Schiphol-Rijk, The Netherlands). Serial two-fold dilutions of serum samples (for IgG, IgG1 and Ig2a) and BAL and nasal wash samples (for IgA determination) were applied to the plates and incubated for 90 min. Plates were washed three times with PBST and incubated with a 1:5000 dilution of horseradish-peroxidase conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA; Southern Biotech, Birmingham, AL, USA) for 60 min at 37°C. Subsequently, the plates were washed three times with PBST and three times with PBS. After aspiration, *o*-Phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA) in 50 mM phosphate buffer pH 5.6 with 0.02% H_2O_2 was added and wells were incubated for 30 min. Then, the reaction was stopped by adding 50 µL 2M H_2SO_4 per well and the optical densities (OD) of the wells at 492 nm was determined. IgA levels were expressed as OD-values of undiluted samples. IgG levels were expressed as titers and defined as the reciprocal of the highest dilution that gave an OD value of at least 0.2.

2.6 Antibody affinity measurement

Antibody affinity was determined in pooled sera. Pooled sera from different mouse groups were adjusted such that they contained a comparable titer of RSV-specific antibodies. These antibodies were first allowed to bind to the wells of RSV-coated ELISA plates and subsequently the wells were washed with different concentrations of urea solution. Then, antibodies that remained bound to the RSV-coated plates were determined by standard methods, as described above. The percentage of bound antibodies was calculated by comparing levels of bound antibodies after normal washes with levels after urea-buffer washes.

2.7 IFN-γ and IL-5 detection in stimulated splenocyte supernatants and BAL

Four days after the virus challenge, mice were sacrificed and spleens were harvested separately in 15 mL tubes containing Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, Breda, The Netherlands) supplemented with 1% Penicillin/Streptomycin and 0.1% beta-mercaptoethanol (Invitrogen, Breda, The Netherlands) and 10% FCS (Lonza-Biowhittaker, Basel, Switzerland). Then, spleens were processed individually for *in vitro* stimulation. Briefly, washed spleens were passed through a 70 µm mesh (BD Biosciences, Heidelberg, Germany) using sterile 3 mL syringe plungers. Subsequently, erythrocytes were lysed by incubating with hypotonic medium (0.83 % NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 5 min on ice. The cells were washed with IMDM, counted and brought to appropriate concentrations. Fresh spleen cells were seeded into 96-well plates at a concentration of 2×10^6 cells/mL and stimulated with BPL-RSV (10 µg/mL) in IMDM/10% FCS in triplicates and incubated at 37°C in a 5% CO_2 atmosphere for 72 h. Supernatants were harvested and stored at –20°C until further analysis.

IFN-γ and IL-5 cytokines were measured in supernatants of these stimulated splenocytes as well in BAL taken at time of sacrifice (4 days after challenge). For this, mouse IFN-γ- and mouse IL-5- high sensitivity ELISA kits (eBioscience, Vienna, Austria) were used according to the manufacturer's instruction. Detection limits were 15 pg/mL and 4 pg/mL for IFN-γ and IL-5, respectively.

2.8 Lung viral titration

Lungs were removed aseptically from all mice following euthanasia and washed in Dulbecco's Modified Essential Medium (DMEM), (PAA Laboratories, Colbe, Germany), supplemented with 2% FCS and transferred into 4 mL tubes containing 1 mL medium. Then, the lungs were homogenized individually with an automated Potter homogenizer Polytron-Aggregate® (Thomas Scientific, Swedesboro, NJ, USA), centrifuged at 1400 rpm for 10 min at 4°C and supernatants were separated. To determine viral titers, $TCID_{50}$ titers were determined. Briefly, HEp-2 cells, 15,000 per well in DMEM with 2% FCS, were seeded in 96-well plates one day before and incubated overnight at 37°C in a 5% CO_2 atmosphere. Next day, supernatants were removed from the plates and washed with PBS. Then, serial dilutions of the lung supernatants were added to the plates and incubated for 5-6 days at 37°C in a 5% CO_2 atmosphere. Then, supernatants were removed and plates were washed with PBS. The cells were then fixed with 1% para-formaldehyde in PBS for 1 h. After blocking cells with 2% skimmed milk in PBS, plates were incubated at RT for 2 h with a 1:400 dilution of FITC-conjugated goat anti-RSV antibody (Meridian life science Inc, Saco, ME, USA) and washed with PBS. Wells were considered positive when ≥ 1 fluorescent syncytium was detected. Finally, $TCID_{50}$ titers were calculated by the Reed-Muench method using an Excel spreadsheet.

2.9 Chemokine mRNA determination in lung through qPCR

Parts of the lungs were collected in 1.5 mL tubes containing 1 mL RNAlater, RNA stabilization reagent, (Qiagen Benelux B.V., Venlo, The Netherlands) and stored at −80°C till further processing. Total RNA was extracted with a Rneasy kit (Qiagen Benelux B.V., Venlo, The Netherlands) according to the manufacturer's instructions and RNA concentration was determined by spectrophotometry. First-strand cDNA was synthesized from equal amounts of RNA with a cDNA synthesis kit (Thermo Scientific (Abgene), Epsom, UK). CCL11 (eotaxin) mRNA levels were determined by real-time PCR analysis with SYBR green Premix Taq (Westburg, Leusden, The Netherlands). Primer sequences used in this study are as follows. ß-actin sense: TAAAGACCTCTATGCCAACACAGT, antisense: CACGATGGAGGGGCCGGACTCTTC; CCL11 sense: TATTCCTG CTGCTCACGGTCACTT,antisense:TCTCTTTGCCCAACCTGGTCTTGA. PCR reactions were performed in a StepOne real time PCR system (Applied

Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) at recommended reaction conditions: 95°C for 15 min, then 40 cycles of denaturation at 95°C for 15 sec, annealing at 50-60°C for 30 sec and extension at 72°C. A melt curve analysis was performed to control the specificity of the amplification products. The gene-specific threshold cycle (C^t) for each sample was corrected by subtracting the Ct for the housekeeping gene ß-actin (ΔC^t). Lung samples from untreated naive mice were chosen as the reference samples and the ΔC^t for all the experiment samples were calculated by subtracting ΔC^t for the reference samples ($\Delta\Delta C^t$). Then, the fold-difference of the test gene mRNA was calculated by using the formula $2^{-\Delta\Delta Ct}$ [27].

2.10 BAL cell cytospot staining

BAL fluids were centrifuged and cell pellets were resuspended in 500 µL PBS. Then, these samples were spun at 300 rpm for 5 min onto glass slides, air dried, and subsequently fixed in 80% methanol/20% PBS (V/V) for 10 min at –20°C. After air drying, slides were stained for 20 min in May-Grunwald-Giemsa stain (Merck, Darmstadt, Germany), diluted 1:1 in Sørensen's phosphate buffer (0.2 M; pH 6.6). Next, slides were rinsed in Sørensen's phosphate buffer, and incubated for 15 min in Giemsa stain (Merck, Darmstadt, Germany) diluted 1:8 in Sørensen's phosphate buffer. After washing with tap water, slides were air dried and spots were sealed using cover slides and Kaiser's glycerin (Merck, Darmstadt, Germany). The presence of eosinophils in cytospot BAL cells was analysed by light microscopy.

2.11 Data analysis

All statistical analyses were performed using Graphpad Prism v3.0 (Graphpad Software, Lajolla, CA, USA). Statistical significance was determined using unpaired Mann-Whitney U test and Wilcoxon matched paired t test. *P* values ≤0.05 were considered statistically significant.

3. Results

3.1 In vitro NF-κB activation by BPL-RSV supplemented with CpG ODN/L18-MDP adjuvants

In order to analyse the possible synergistic activity of L18-MDP, CpG ODN and inactivated RSV, i.e. BPL-RSV, on activation of antigen-presenting cells (APC), NF-κB induction in a reporter APC (RAW-Blue™ cells) was measured (Figure 1). L18-MDP alone poorly induced NF-κB. Similarly, BPL-RSV alone in the absence of adjuvants did not result in significant NF-κB induction either (Figure 1A), even not at higher concentrations (data not shown). L18-MDP combined with BPL-RSV, however, resulted in a synergistically enhanced NF-κB activation (Figure 1A). CpG ODN gave a dose-dependent

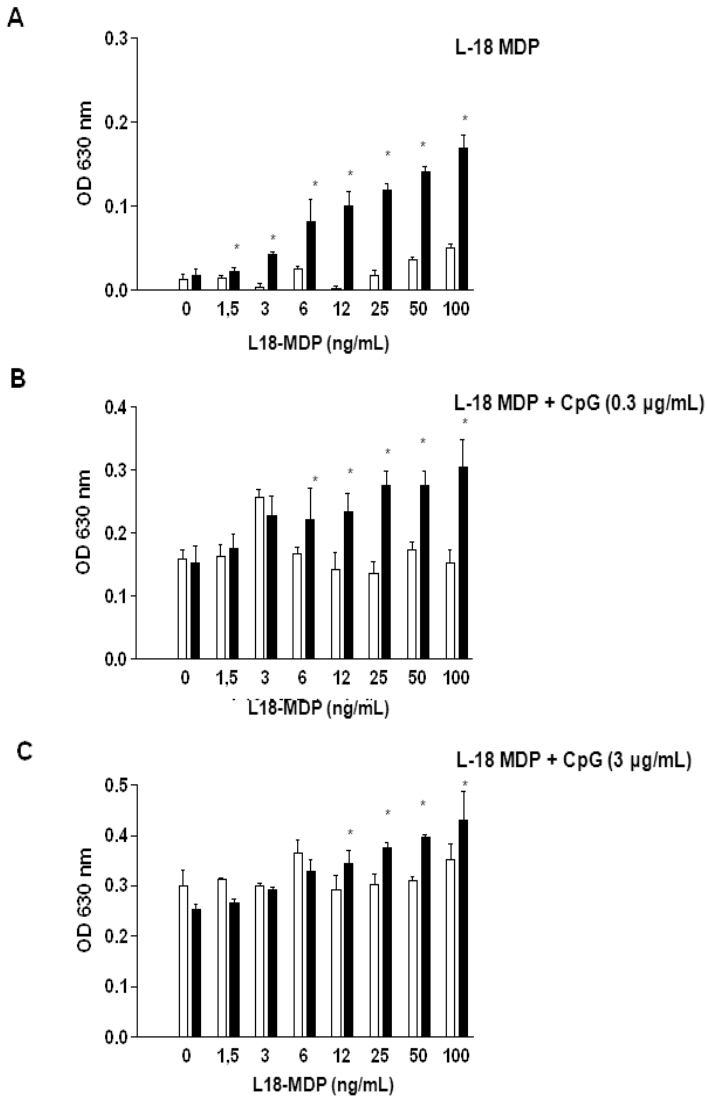

Figure. 1. Efficient NF-κB activation by L18-MDP *in vitro* occurs only in presence of RSV and is further enhanced by CpG ODN. Reporter gene transfected-antigen-presenting cells were activated with different concentrations of CpG ODN/L18-MDP alone or added to a fixed concentration of RSV (10 µg/mL). NF-κB activation was read out by a colorimetric assay measuring substrate cleavage by alkaline phosphatase, secreted after induction through the NF-κB responsive reporter gene. Open bars represent means ± SD of cells stimulated with innate receptor ligands at different concentrations in the absence of RSV and black bars represent means ± SD of cells stimulated with innate receptor ligands at different concentrations in the presence of RSV (10 µg/mL). An asterisk indicates a statistically significant difference ($p \leq 0.05$; Mann Whitney U test) between responses in the absence and presence of RSV particles.

activation of NF-κB which could be enhanced by adding L18-MDP but, again, only when viral particles were present (Figure 1B, 1C). Addition of L18-MDP to CpG ODN generally resulted in increased NF-κB responses, but not in a synergistic way (Figure 1B, 1C). So, our *in vitro* data point towards a synergistic induction of NF-κB by combining BPL-RSV and L18-MDP together. We further tested combinations of BPL-RSV with CpG ODN and L18-MDP in mice using different methods of local administration.

3.2 Induction of systemic IgG and mucosal IgA responses after intranasal (IN) or total respiratory tract (TRT) immunization

First, mice were immunized IN with vaccine formulations containing BPL-RSV alone or BPL-RSV supplemented with CpG ODN alone or both CpG ODN and L18-MDP or PBS (control group) One week after the booster immunization, RSV-specific IgA was detectable only in BAL and nasal washes of mice that received RSV plus adjuvant (Figure 2B, C). With supplementation of L18-MDP to the formulation of BPL-RSV and CpG ODN, a higher proportion of mice had a detectable IgA response in the BAL. RSV-specific serum IgG levels were low or undetectable in all mice (Figure 2A). Only in the group immunized with BPL-RSV supplemented with both CpG ODN and L18-MDP, IgG levels were slightly, but significantly, higher than in naïve mice ($p<0.05$; Figure 2A). As combined CpG ODN, L18-MDP and inactivated viral particles appeared most efficient *in vitro* and *in vivo*, we further tested this combination in mice comparing different methods of local administration to the respiratory tract.

To this end, mice were immunized IN or through total respiratory tract (TRT) immunization using BPL-RSV only or BPL-RSV supplemented with CpG ODN/L18-MDP ligands. TRT immunization with BPL-RSV vaccine supplemented with CpG ODN/L18-MDP ligands induced significantly higher IgG antibody responses compared to BPL-RSV vaccine alone ($p<0.002$; Figure 3A). IN immunization was less effective in IgG induction (Figure 3A). In order to analyse the phenotype of the immune response, Th1-signature IgG subtype (IgG2a) and Th2-signature IgG subtype (IgG1) antibodies were determined. Supplementation of BPL-RSV vaccine with CpG ODN/L18-MDP increased IgG2a responses, but not IgG1 responses after TRT immunization ($p<0.002$; Figure 3B). This effect was not seen after IN vaccination. Thus, BPL-RSV vaccine supplemented with TLR9/NOD2 ligands augments Th1-signature IgG2a subtype responses upon mucosal immunization, particularly after TRT immunization.

In order to determine mucosal immune responses, mice were immunized by IN or TRT inoculation of BPL-RSV vaccine with and without CpG ODN/L18-MDP as described above. Four days after RSV challenge, mice were sacrificed and nasal wash and BAL samples were taken for analysis of IgA antibody levels. We found significantly increased BAL and nasal IgA levels in mice immunized through the TRT route with BPL-RSV supplemented with CpG ODN/L18-MDP compared to mice immunized with BPL-RSV only ($p<0.004$ and $p<0.02$ for nasal and BAL IgA, respectively; Figure 4). BAL and nasal IgA was induced in some of the mice that were immunized IN with

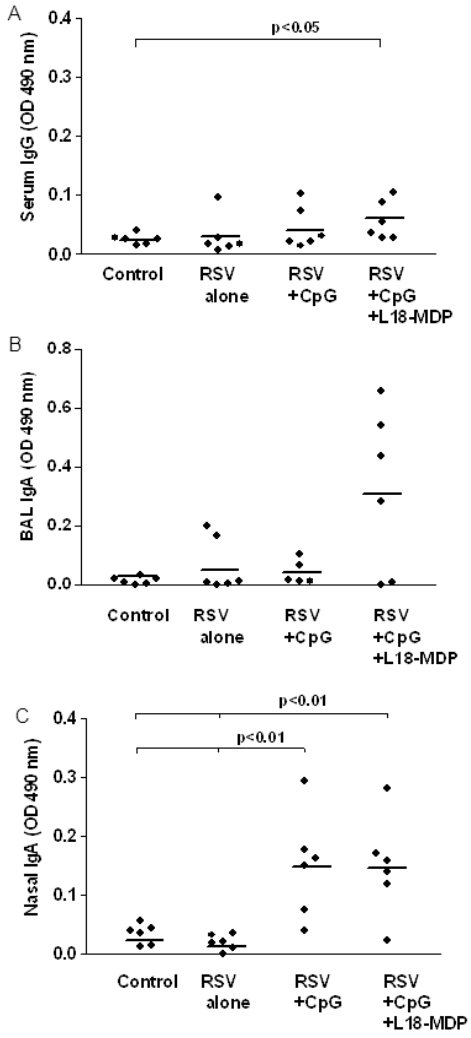

Figure 2. RSV-specific systemic IgG antibody responses after IN immunization. BALB/c mice were immunized IN with vaccine formulations containing 5 µg of BPL-RSV alone, 5 µg BPL-RSV supplemented with CpG ODN (4 µg) and 5 µg BPL-RSV supplemented with CpG ODN (4 µg) and L18-MDP (0.1 µg) or PBS (control group), on day 0 and 21. One week after the booster immunization, RSV-specific IgG responses in serum (A), IgA in BAL (B) and IgA in nasal washes (C) were determined by ELISA. Data from individual mice are shown. The data shown are representative data of at least 2 separate experiments. Data was analysed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.

BPL-RSV supplemented with CpG ODN/L18-MDP adjuvants, but not in mice immunized IN with BPL-RSV alone. Thus, BPL-RSV vaccine together with TLR9/NOD2 ligands induces local IgA responses upon mucosal immunization, particularly after TRT immunization.

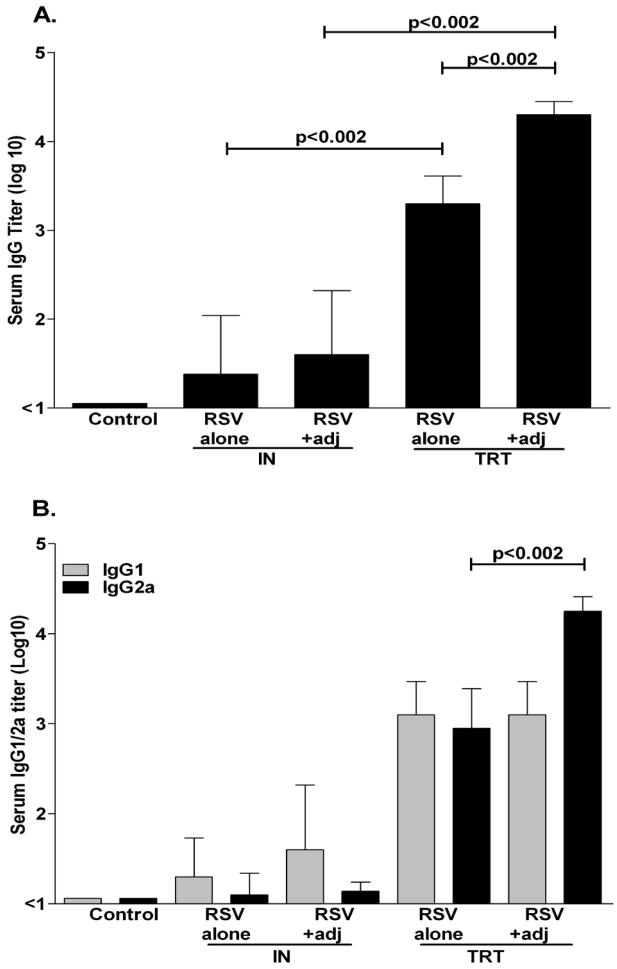

Figure 3. RSV-specific systemic IgG antibody responses after IN or TRT immunization. BALB/c mice were immunized with vaccine formulations containing 5 µg of BPL-RSV alone, 5 µg BPL-RSV supplemented with CpG ODN (4 µg) and L18-MDP (0.1 µg) or PBS (control group), on day 0 and 21. One week after the booster immunization, RSV-specific IgG responses in serum (A) and IgG-subtypes IgG1 and IgG2a (B) were determined by ELISA. Panel A&B: Bars represent the geometric mean titer and standard deviation. The data shown are representative data of at least 2 separate experiments. Data was analysed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.

3.3 Antibody affinity measurement

Next, we investigated if supplementation of BPL-RSV with TLR9/NOD2 ligands could increase the affinity maturation of the RSV-specific IgG antibodies induced by mucosal immunization. The affinity was determined by affinity ELISA, as described by others [8]. Pooled serum antibodies from

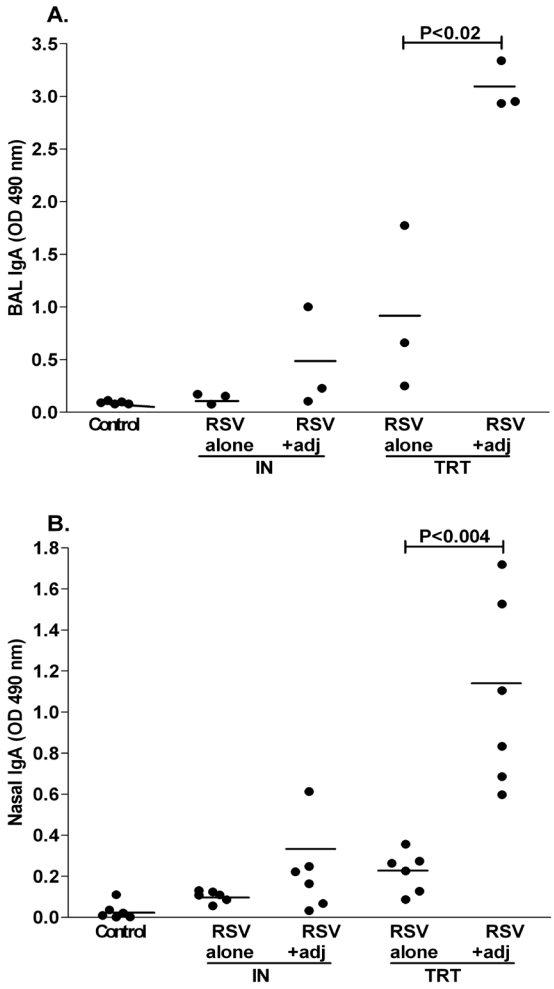

Figure 4. RSV-specific mucosal IgA antibody responses after IN or TRT immunization. BALB/c mice were immunized with vaccine formulations containing 5 µg of BPL-RSV alone, BPL-RSV supplemented with CpG ODN (4 µg) and L18-MDP (0.1 µg) or PBS (control), on day 0 and 21. Four days after challenge with live RSV (day 32), RSV-specific IgA responses in BAL (A) and nasal washes (B) were determined by ELISA. Panel A&B: Each dot represents data from an individual mouse and horizontal line depicts the mean absorbance at 490 nm. The data shown are representative data of at least 2 separate experiments. Data was analysed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.

mice TRT-vaccinated with BPL-RSV supplemented with CpG ODN/L18-MDP showed a higher binding capacity than pooled serum antibodies from mice that had received BPL-RSV only (Figure 5). Therefore, TLR9/NOD2 ligands promote affinity maturation of RSV-specific IgG antibodies after mucosal immunization.

3.4 RSV-specific cell-mediated immune responses

To further characterize the phenotype of the cellular immune response, RSV-specific cytokine responses by splenocytes were measured. Increased secretion of IFN-γ was seen in splenocyte cultures from mice immunized with BPL-RSV supplemented with CpG ODN/L18-MDP compared to cultures from mice that received BPL-RSV alone ($p<0.004$ and $p<0.002$ for IN and TRT immunization, respectively; Figure 6A). The highest production of IFN-γ was seen after TRT immunization. In parallel, IL-5 production

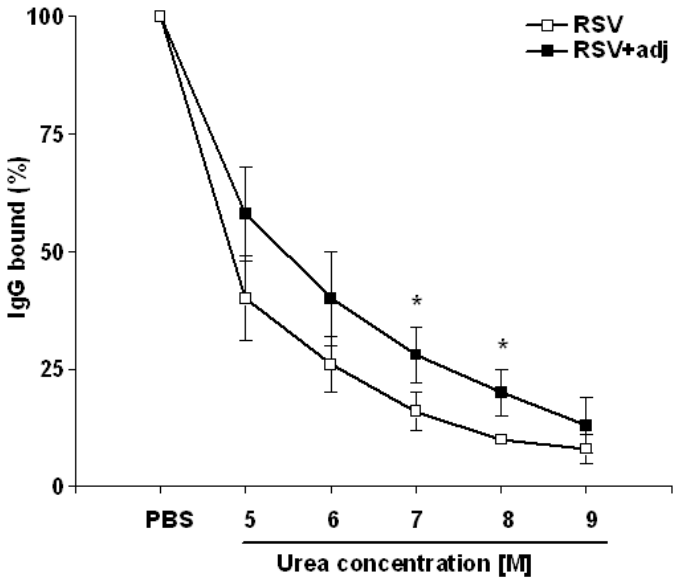

Figure 5. Supplementation of BPL-RSV with CpG ODN/L18-MDP increases IgG antibody affinity. IgG antibody affinity was determined in pooled sera from mice immunized with BPL-RSV alone or BPL-RSV supplemented with CpG ODN/L18-MDP. Serum IgG bound to RSV in ELISA plates was washed with different concentration of urea followed by detection of antibody using standard ELISA procedures. Each point represents the mean percentage ± SD of antibodies that remained bound after washes with different urea concentrations. The SD represents triplicates of the pooled sera. The data shown is representative of 3 separate measurements. An asterisk indicates a statistically significant difference ($p \leq 0.05$; Wilcoxon matched paired t test).

was significantly decreased ($p<0.002$ and $p<0.004$ for IN and TRT immunization, respectively; Figure 6B). This was seen irrespective of the method of mucosal immunization, i.e. IN or TRT immunization. To know if splenocyte responses reflect local responses during lung infection with RSV, levels of IL-5 and IFN-γ and, particularly their ratio, was also determined in lung washes from immunized and IN-infected mice. Here, the ratios of IFN-γ to IL-5 in lungs of mice given BPL-RSV/CpG ODN/L18-MDP by TRT immunization were specifically compared with ratios determined in the lungs of mice immunized IM with FI-RSV. The IFN-γ to IL-5 ratio was significantly increased in mice that previously were TRT-immunized with BPL-RSV/CpG ODN/L18-MDP compared to the ratios determined in naïve (buffer) or FI-RSV-immunized mice ($p<0.05$ and $p<0.01$ for buffer and FI-RSV groups, respectively; Figure 6C). Therefore, CpG ODN/L18-MDP supplemented BPL-RSV efficiently primes for Th1-phenotype skewed responses, not only in recall responses of RSV-stimulated splenocytes but also in local responses in the lung upon infection with RSV.

3.5 Protection from live RSV challenge

In addition to immune parameters, protection against viral infection was measured. For this, mice were IN or TRT immunized with BPL-RSV alone or BPL-RSV supplemented with CpG ODN and L18-MDP on day 0 and 21. One week after the second immunization, mice were infected by IN inoculation of live virus under anesthesia and 4 days later, lung viral titers were measured. Mice that received inactivated RSV by TRT immunization showed significant lower viral titers compared to titers seen in the non-vaccinated control group. Notably, mice that received BPL-RSV supplemented with CpG ODN/L18-MDP through TRT immunization had significantly decreased viral titers compared to mice receiving BPL-RSV only using the same route ($p<0.01$; Figure 7). IN-immunized mice also showed reduced viral titers compared to titers in non-immune control mice, but only when immunized with BPL-RSV supplemented with adjuvants ($p<0.01$; Figure 7). These data show that supplementation of mucosally-delivered BPL-RSV with TLR9/NOD2 ligands increases its capacity to induce protective immune responses against live RSV.

3.6 Eotaxin production and eosinophil influx in lungs of infected mice

In order to monitor if the induced protective immunity was paralleled with a possible priming for enhanced disease, lung mRNA levels for a chemokine associated with enhanced disease, e.g. CCL11 (eotaxin), were determined 4-days after infectious virus challenge. Supplementation of BPL-RSV with CpG ODN/L18-MDP ligands significantly reduced lung CCL11 mRNA levels (Figure 8). This was observed not only after TRT immunization but also after IN immunization (Figure 8).

The previous data showed that TRT immunization with BPL-RSV supplemented with CpG ODN/L18-MDP is the most optimal approach to induce high RSV-specific serum IgG and mucosal IgA antibodies, Th1-type

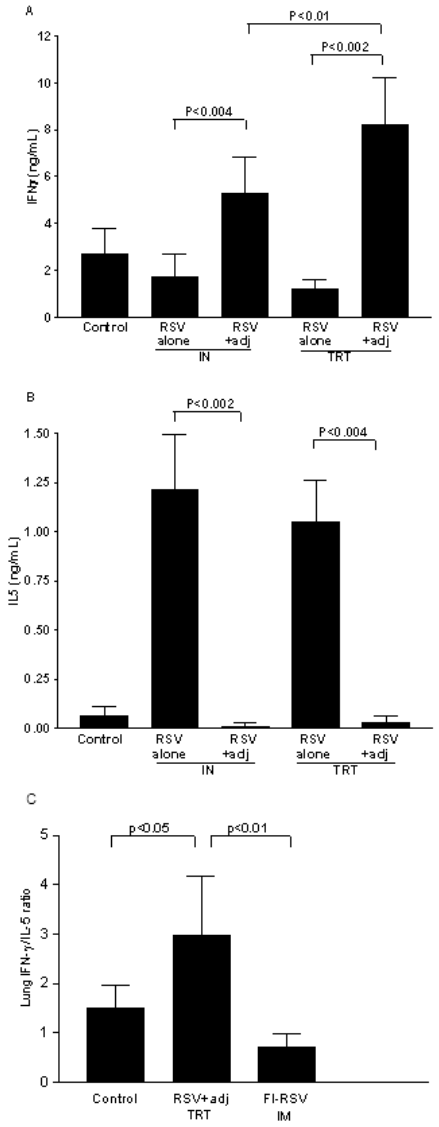

Figure 6. *Ex vivo* cytokine production by splenocytes in response to stimulation with RSV. IFN-γ (Panel A) and IL-5 (Panel B) production in splenocyte cultures after stimulation with BPL-RSV was determined by cytokine ELISA. Cytokines in the culture supernatants were assayed after 3 days of culturing. Lung cytokines in BAL of immunized mice were measured 4 days after challenge and their ratio was calculated (Panel C). Bars and error bars represent means ± SD. The data shown are representative data of at least 2 separate experiments. Data was analysed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.

responses and protection against infection without induction of lung CCL11 mRNA. To confirm that immunization with this formulation does not lead to influx of eosinophils into the lungs, cytospots of BAL cells obtained after challenge were analysed. They were specifically compared with BAL cells obtained from mice that were immunized IM with alum-absorbed FI-RSV, as this vaccine formulation is known to prime for immune responses that lead to considerable influx of eosinophils into the lung upon infection. Indeed, mice vaccinated with FI-RSV showed eosinophil influx into their lungs (41 ± 7, mean % ± SD of all BAL cells). In contrast, no eosinophils were detected in BAL of TRT-immunized mice with BPL-RSV supplemented with CpG ODN/L18-MDP, after challenge (0 ± 0, mean % ± SD of all BAL cells). Thus, mucosal immunization with BPL-RSV supplemented with CpG ODN/L18-MDP does not prime eosinophil influx, i.e. enhanced disease.

4. Discussion

Efforts to develop an effective inactivated RSV vaccine have as yet been unsuccessful, in part due to the disastrous outcome of earlier clinical trials with FI-RSV vaccine among naive children in the 1960s [3]. Animal studies later suggested that this type of inactivated RSV vaccine induces low-affinity antibodies and Th2-biased immune responses in immunized animals which fail to protect but can augment immunopathology upon natural infection [5]. In the present study, we investigated the combined use of a model inactivated RSV vaccine, i.e. BPL-RSV, supplemented with TLR9/NOD2 ligands, with a mucosal route of vaccine administration. Our results suggest that this is a safe and effective approach to induce RSV-specific immunity. Our data show that inactivated viral particles combined with CpG ODN/L18-MDP potently activate NF-κB in a model APC system *in vitro*. *In vivo*, this vaccine formulation also induced local IgA responses and high-affinity serum IgG and Th1-signature IgG2a antibody responses upon mucosal immunization, particularly after TRT immunization. Co-administered CpG ODN/L18-MDP shifted cellular immune responses towards a dominant IFN-γ-producing Th1-type response. Finally, protection against infection was conferred without priming for enhanced disease. Therefore, mucosal immunization with inactivated RSV, supplemented with innate receptor ligands, appears to be a promising and safe approach to induce an effective RSV-specific immune response.

Previous studies used TLR ligand-supplemented RSV to reduce priming for enhanced disease. For example, Johnson *et al.* showed that supplementation of CpG ODN to IM-injected FI-RSV could reduce lung pathology upon RSV infection of immunized mice [17]. This was paralleled with a more Th1-skewed response and lowered eotaxin expression in the lungs of infected mice. In line with these data, we show that mucosal immunization with a CpG ODN-containing RSV vaccine also leads to Th1-skewed responses and lowered eotaxin expression in the lungs (Figure 6 & 8). Prince *et al.*, however showed that supplementation of RSV F protein

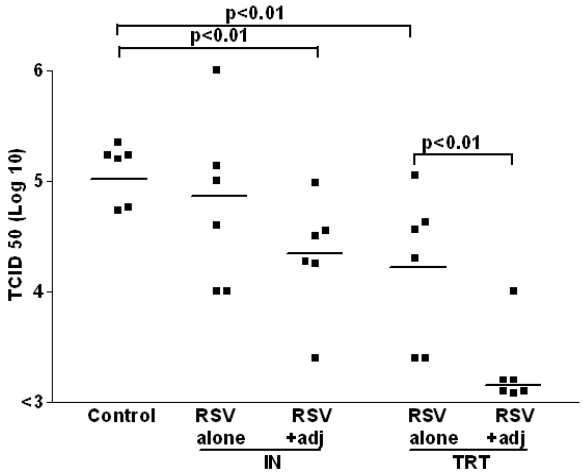

Figure 7. Protection of mice from challenge with live virus. BALB/c mice were immunized with vaccine formulations containing 5 µg of BPL-RSV alone, BPL-RSV supplemented with CpG ODN (4 µg) and L18-MDP (0.1 µg) or PBS (control group), on day 0 and 21. Four days after challenge with live RSV (day 32), lung viral titers were determined. Viral titers are expressed as $TCID_{50}$. Each point represents data from an individual mouse. The data shown are representative data of at least 2 separate experiments. Data was analysed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.

with CpG ODN increased lung pathology in cotton rats [28]. Possibly, cotton rat respond differently to CpG ODN than mice, the animal model used in our study and that of Johnson *et al.* Alternatively, it could be due to the CpG ODN type used. Although Prince *et al.* showed that their CpG ODN induced cotton rat splenocyte proliferation, it was not investigated if it induces cytokines that are important inducers of Th1-type responses, like IL-12 which is readily induced by the CpG ODN type used in our study and that of Johnson *et al.* i.e. CpG ODN 1826 [29].

Administration of BPL-RSV through TRT immunization resulted in a more efficient induction of immune responses compared to administration of RSV antigen to the nasal cavity only, i.e. IN immunization. In anesthetized mice, a small inoculation volume reaches the nasal cavity only, while a large inoculation volume targets both the nasal cavity and total respiratory tract, including the lungs [20,25]. A number of factors have been described that contribute to a lower immunization efficacy when targeting the nasal

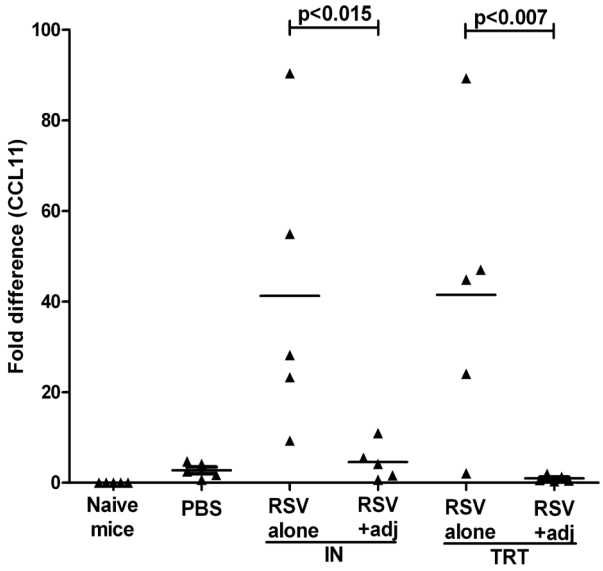

Figure. 8. CCL11 chemokine mRNA levels in RSV-infected lungs. BALB/c mice were immunized according to standard procedures and mouse lungs were collected 4 days after challenge. Total mRNA was isolated and qPCR was performed after cDNA synthesis, to determine levels of CCL11 (eotaxin) mRNA. CCL11 levels in lungs of untreated naive mice were subtracted from the levels detected in lungs of immunized groups. Each point represents data from an individual mouse. The data shown are representative data of at least 2 separate experiments. Data was analysed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.

cavity. These factors include a low retention time of inoculated vaccine at this site, lack of uptake of vaccine antigens through M cells or penetration of vaccine antigens through the nasal epithelium [30]. Although mucosal administration of antigen can induce protective immunity, it may also lead to mucosal tolerance [31,32]. Our data showed that mice that received BPL-RSV which was confined to the nasal cavity only (IN immunization) primed RSV-specific T cell immunity, with particularly IL-5 secretion. Addition of innate receptor ligands to the antigen clearly abrogated this Th2-type response and skewed responses towards a Th1-phenotype (Figure 6). However, supplementation of BPL-RSV with innate receptor ligands did not clearly stimulate mucosal and systemic antibody responses when the inoculum was targeted to the nasal cavity only.

Vaccine administered through TRT immunization appears to be more efficient in inducing effector-type immune responses, and preventing tolerogenic responses [33]. Indeed, our data show that efficient local and systemic antibody responses are induced after TRT immunization, but not

after IN immunization, which might be due to deposition of antigen in the lungs, an environment less prone to induction of tolerogenic responses [33]. Furthermore, it is likely that there is a slower clearance of vaccine antigen from the lungs as compared to the nasal cavity. Thus, our data confirm the findings of others [33] that the lungs are a better site for induction of combined mucosal and systemic immune responses as compared to the nasal cavity. It is likely that activation of TLR9 and NOD2 receptors in alveolar macrophages and lung dendritic cells by the CpG ODN/L18-MDP is important in the induction of the IgA response, in a similar fashion as virus like particles that, through ssRNA activation of TLR7 on these cells, enhance local IgA responses [34].

Innate ligands, particularly TLR9 ligands like CpG ODN 1826, proved to be effective in enhancement of class-switch recombination to IgA and mucosal IgA responses [22,34]. It has been reported that genital tract IgA antibody-secreting cells (ASC) were increased in number after mucosal immunization of mice with recombinant glycoprotein B of herpes simplex virus supplemented with CpG ODN [22]. This enhanced IgA ASC response may be due to the ability of CpG ODN to directly activate B cells, macrophages and dendritic cells [35-37]. Besides CpG ODN, also NOD2 ligands, like L18-MDP can directly enhance B cell receptor-induced B cell activation in the absence of T cell help [38]. The latter studies suggest that, besides T cell-dependent pathways toward class-switch recombination to, for example, IgA, T cell-independent pathways exist that are initiated through innate receptor signaling. TLR activation, for example, induces factors inducing cytokines such as B-lymphocyte-Activating Factor belonging to the TNF superfamily (BAFF) and A-Proliferation-Inducing-Ligand (APRIL) [34,39]. A recent study reported that TLR ligands, i.e. bacterial RNA, deposited in the respiratory tract augment antibody responses by secreting BAFF and APRIL cytokines by activated lung DCs and macrophages [34]. Upon binding of DC or macrophage-derived BAFF to receptors on B cells, i.e. BAFF-R or Transmembrane Activator and Calcium-modulator and cyclophilin-ligand Interactor (TACI), this factor co-stimulates B cell proliferation, Ig secretion and class-switch recombination towards, for example, IgA [40-42]. Interestingly, BAFF is produced by lung dendritic cells and alveolar macrophages upon contact with different TLR ligands, including TLR7, TLR9 and TLR3 ligands [34,41,43]. BAFF may also be induced upon TRT immunization with BPL-RSV supplemented with CpG ODN/L18-MDP and could contribute to the induction of local IgA responses. Thus, co-administered innate receptor ligands induce potent IgA and IgG antibodies which might be due to activation and proliferation of B cells and possibly linked with secretion of BAFF/APRIL cytokines by lung DC and alveolar macrophages.

Untill now, the relative efficacy of IgA versus IgG antibody in terms of RSV-neutralizing capacity has remained unclear. Although a small contribution of adjuvants on the innate immunity-mediated protection is possible, RSV-specific IgG antibodies (Figure 3) showed a clear negative correlation with lung viral titers (Figure 7; Spearman r -0.5965, p=0.0003), suggesting that antibodies are the main contributors to protection. In line with these data, RSV-specific IgG, either induced by parenteral vaccination,

administered as a monoclonal antibody through intraperitoneal (IP) injection, or inoculated IN, can protect mice against a TRT challenge [18,44]. Potent RSV-neutralizing capacity of IgA has also been demonstrated in studies that either studied protective effects of IN-inoculated RSV-specific IgA monoclonal antibody or adenoviral vector-based RSV vaccine induced mucosal IgA [45-47]. This suggests that the presence and level of RSV-specific antibodies in the respiratory tract, either locally produced or transudated from the serum to the mucosa, is more important than the isotype of the RSV-specific antibody in protecting the lung from challenge with live virus of the TRT. It is likely that local production of RSV-specific antibody, either IgG or IgA, would significantly contribute to protection upon infection [48], particularly after natural infection which is likely to start in the upper respiratory tract. Thus, optimal protective immunity against RSV would probably need to consist of both serum IgG as well as locally produced antibodies, including IgG and IgA.

We conclude that mucosal immunization with inactivated RSV antigen supplemented with TLR9/NOD2 ligands is a promising approach to induce RSV-specific immunity. Mucosal immunization of the lower respiratory tract has become feasible with the recent development of dry powder inhalation specifically developed for vaccination [49,50]. This approach could further be explored by using non-replicating RSV vaccine candidates like, for example, RSV virosomes which can easily be equipped with lipophilic TLR and/or NOD2 ligands [18].

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6. References

1. Dowell SF, Anderson LJ, Gary HE,Jr., Erdman DD, Plouffe JF, File TM,Jr. et al. Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults. J Infect Dis 1996;174(3):456-62.
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(17):1749-59.
3. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89(4):422-34.
4. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol Rev 2011;239(1):149-66.
5. 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(5):2852-60.
6. Culley FJ, Pennycook AM, Tregoning JS, Hussell T, Openshaw PJ. Differential chemokine expression following respiratory virus infection reflects Th1- or Th2-biased immunopathology. J Virol 2006;80(9):4521-7.
7. Miller AL, Bowlin TL, Lukacs NW. Respiratory syncytial virus-induced chemokine production: linking viral replication to chemokine production in vitro and in vivo. J Infect Dis 2004;189(8):1419-30.
8. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 2009;15(1):34-41.
9. Hussell T, Humphreys IR. Nasal vaccination induces protective immunity without immunopathology. Clin Exp Immunol 2002;130(3):359-62.
10. Singleton R, Etchart N, Hou S, Hyland L. Inability to evoke a long-lasting protective immune response to respiratory syncytial virus infection in mice correlates with ineffective nasal antibody responses. J Virol 2003;77(21):11303-11.
11. Mapletoft JW, Latimer L, Babiuk LA, van Drunen Littel-van dH. Intranasal immunization of mice with a bovine respiratory syncytial virus vaccine induces superior immunity and protection compared to those by subcutaneous delivery or combinations of intranasal and subcutaneous prime-boost strategies. Clin Vaccine Immunol 2010;17(1):23-35.
12. Kimman TG, Westenbrink F, Schreuder BE, Straver PJ. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. J Clin Microbiol 1987;25(6):1097-106.
13. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 1991;163(4):693-8.
14. Taylor CE, Craft AW, Kernahan J, Millman R, Reid MM, Scott R et al. Local antibody production and respiratory syncytial virus infection in children with leukaemia. J Med Virol 1990;30(4):277-81.
15. Belyakov I.M. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens. Journal of immunolgy 2009.
16. Boukhvalova MS, Prince GA, Soroush L, Harrigan DC, Vogel SN, Blanco JC. The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. Vaccine 2006;24(23):5027-35.
17. Johnson TR, Rao S, Seder RA, Chen M, Graham BS. TLR9 agonist, but not TLR7/8, functions as an adjuvant to diminish FI-RSV vaccine-enhanced disease, while either agonist used as therapy during primary RSV infection increases disease severity. Vaccine 2009;27(23):3045-52.
18. 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(34):5543-50.
19. Tada H, Aiba S, Shibata K, Ohteki T, Takada H. Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and

T helper type 1 cells. Infect Immun 2005;73(12):7967-76.
20. McCluskie MJ, Weeratna RD, Davis HL. Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. Mol Med 2000;6(10):867-77.
21. Fukushima A, Yoo YC, Yoshimatsu K, Matsuzawa K, Tamura M, Tono-oka S et al. Effect of MDP-Lys(L18) as a mucosal immunoadjuvant on protection of mucosal infections by Sendai virus and rotavirus. Vaccine 1996;14(6):485-91.
22. Gallichan WS, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. J Immunol 2001;166(5):3451-7.
23. Lin L, Gerth AJ, Peng SL. CpG DNA redirects class-switching towards "Th1-like" Ig isotype production via TLR9 and MyD88. Eur J Immunol 2004;34(5):1483-7.
24. Ogawa T, Shimauchi H, Hamada S. Mucosal and systemic immune responses in BALB/c mice to Bacteroides gingivalis fimbriae administered orally. Infect Immun 1989;57(11):3466-71.
25. Southam DS, Dolovich M, O'Byrne PM, Inman MD. Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. Am J Physiol Lung Cell Mol Physiol 2002;282(4):L833-9.
26. De Haan A, Geerligs HJ, Huchshorn JP, van Scharrenburg GJ, Palache AM, Wilschut J. Mucosal immunoadjuvant activity of liposomes: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes. Vaccine 1995;13(2):155-62.
27. Yuan JS, Reed A, Chen F, Stewart CN, Jr. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006;7:85.
28. Prince GA, Mond JJ, Porter DD, Yim KC, Lan SJ, Klinman DM. Immunoprotective activity and safety of a respiratory syncytial virus vaccine: mucosal delivery of fusion glycoprotein with a CpG oligodeoxynucleotide adjuvant. J Virol 2003;77(24):13156-60.
29. Jakob T, Walker PS, Krieg AM, von Stebut E, Udey MC, Vogel JC. Bacterial DNA and CpG-containing oligodeoxynucleotides activate cutaneous dendritic cells and induce IL-12 production: implications for the augmentation of Th1 responses. Int Arch Allergy Immunol 1999;118(2-4):457-61.
30. Amidi M, Romeijn SG, Verhoef JC, Junginger HE, Bungener L, Huckriede A et al. N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model. Vaccine 2007;25(1):144-53.
31. Wolvers DA, van dC, Kraal G. Mucosal tolerance is associated with, but independent of, up-regulation of Th2 responses. Immunology 1997;92(3):328-33.
32. Mestecky J, Moldoveanu Z, Elson CO. Immune response versus mucosal tolerance to mucosally administered antigens. Vaccine 2005;23(15):1800-3.
33. Balmelli C, Demotz S, cha-Orbea H, De GP, Nardelli-Haefliger D. Trachea, lung, and tracheobronchial lymph nodes are the major sites where antigen-presenting cells are detected after nasal vaccination of mice with human papillomavirus type 16 virus-like particles. J Virol 2002;76(24):12596-602.
34. Bessa J, Jegerlehner A, Hinton HJ, Pumpens P, Saudan P, Schneider P et al. Alveolar macrophages and lung dendritic cells sense RNA and drive mucosal IgA responses. J Immunol 2009;183(6):3788-99.
35. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci USA 1996;93(7):2879-83.
36. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374(6522):546-9.
37. Yi AK, Hornbeck P, Lafrenz DE, Krieg AM. CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. J Immunol 1996;157(11):4918-25.
38. Petterson T, Jendholm J, Mansson A, Bjartell A, Riesbeck K, Cardell LO. Effects of NOD-like receptors in human B lymphocytes and crosstalk between NOD1/NOD2 and Toll-like

receptors. J Leukoc Biol 2011;89(2):177-87.
39. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol 2002;3(9):822-9.
40. Craxton A, Magaletti D, Ryan EJ, Clark EA. Macrophage- and dendritic cell--dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. Blood 2003;101(11):4464-71.
41. Katsenelson N, Kanswal S, Puig M, Mostowski H, Verthelyi D, Akkoyunlu M. Synthetic CpG oligodeoxynucleotides augment. Eur J Immunol 2007;37(7):1785-95.
42. Nardelli B, Belvedere O, Roschke V, Moore PA, Olsen HS, Migone TS et al. Synthesis and release of B-lymphocyte stimulator from myeloid cells. Blood 2001;97(1):198-204.
43. Xu W, Santini PA, Matthews AJ, Chiu A, Plebani A, He B et al. Viral double-stranded RNA triggers Ig class switching by activating upper respiratory mucosa B cells through an innate TLR3 pathway involving BAFF. J Immunol 2008;181(1):276-87.
44. Fisher RG, Crowe JE,Jr., Johnson TR, Tang YW, Graham BS. Passive IgA monoclonal antibody is no more effective than IgG at protecting mice from mucosal challenge with respiratory syncytial virus. J Infect Dis 1999;180(4):1324-7.
45. Weltzin R, Hsu SA, Mittler ES, Georgakopoulos K, Monath TP. Intranasal monoclonal immunoglobulin A against respiratory syncytial virus protects against upper and lower respiratory tract infections in mice. Antimicrob Agents Chemother 1994;38(12):2785-91.
46. Weltzin R, Traina-Dorge V, Soike K, Zhang JY, Mack P, Soman G et al. Intranasal monoclonal IgA antibody to respiratory syncytial virus protects rhesus monkeys against upper and lower respiratory tract infection. J Infect Dis 1996;174(2):256-61.
47. Kim S, Jang JE, Yu JR, Chang J. Single mucosal immunization of recombinant adenovirus-based vaccine expressing F1 protein fragment induces protective mucosal immunity against respiratory syncytial virus infection. Vaccine 2010;28(22):3801-8.
48. Valosky J, Hishiki H, Zaoutis TE, Coffin SE. Induction of mucosal B-cell memory by intranasal immunization of mice with respiratory syncytial virus. Clin Diagn Lab Immunol 2005;12(1):171-9.
49. De Swart RL, LiCalsi C, Quirk AV, van Amerongen G, Nodelman V, Alcock R et al. Measles vaccination of macaques by dry powder inhalation. Vaccine 2007;25(7):1183-90.
50. Sou T, Meeusen EN, de Veer M, Morton DA, Kaminskas LM, McIntosh MP. New developments in dry powder pulmonary vaccine delivery. Trends Biotechnol 2011;29(4):191-8.

Chapter 3

Evaluation of an intranasal virosomal vaccine against Respiratory Syncytial Virus in mice: Effect of TLR2 and NOD2 ligands on induction of systemic and mucosal immune responses

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Abstract

Introduction: RSV infection remains a serious threat to newborns and the elderly. Currently, there is no vaccine available to prevent RSV infection. A mucosal RSV vaccine would be attractive as it could induce mucosal as well as systemic antibodies, capable of protecting both the upper and lower respiratory tract. Previously, we reported on a virosomal RSV vaccine for intramuscular injection with intrinsic adjuvant properties mediated by an incorporated lipophilic Toll-like receptor 2 (TLR2) ligand. However, it has not been investigated whether this virosomal RSV vaccine candidate would be suitable for use in mucosal immunization strategies and if additional incorporation of other innate receptor ligand, like NOD2 ligand, could further enhance the immunogenicity and protective efficacy of the vaccine.

Objective: To explore if intranasal (IN) immunization with a virosomal RSV vaccine, supplemented with TLR2 and/or NOD2 ligands, is an effective strategy to induce RSV-specific immunity.

Methods: We produced RSV-virosomes carrying TLR2 (Pam_3CSK_4) and/or NOD2 (L18-MDP) ligands. We tested the immunopotentiating properties of these virosomes *in vitro*, using TLR2 and/or NOD2 ligand-responsive murine and human cell lines, and *in vivo* by assessing induction of protective antibody and cellular responses upon IN immunization of BALB/c mice.

Results: Incorporation of Pam_3CSK_4 and/or L18-MDP potentiates the capacity of virosomes to activate (antigen-presenting) cells *in vitro*, as demonstrated by NF-κB induction. *In vivo*, incorporation of Pam_3CSK_4 in virosomes boosted serum IgG antibody responses and mucosal antibody responses after IN immunization. While L18-MDP alone was ineffective, incorporation of L18-MDP in Pam_3CSK_4-carrying virosomes further boosted mucosal antibody responses. Finally, IN immunization with adjuvanted virosomes, particularly Pam_3CSK_4/L18-MDP-adjuvanted-virosomes, protected mice against infection with RSV, without priming for enhanced disease.

Conclusion: Mucosal immunization with RSV virosomes, supplemented with incorporated TLR2 and/or NOD2 ligands, represents a promising approach to induce effective and safe RSV-specific immunity.

1. Introduction

Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract infections, particularly in infants and children. According to the WHO reports, RSV causes 64 million infections annually, leading to the hospitalization of 18,000 - 75,000 children in the USA alone with an estimated mortality of 160,000 [1]. Most children are infected at least once by the age of 2 and reinfection may occur throughout life due to incomplete immunity to RSV [2,3]. RSV, therefore, remains a threat at older age, particularly in risk groups such as the elderly and immuno-compromised individuals. Despite the burden of RSV disease, there is still no licensed vaccine against RSV infection.

New candidate non-replicating RSV vaccines should induce protective immunity without priming for enhanced respiratory disease (ERD) upon natural infection, as did the formalin-inactivated and alum-adjuvanted whole RSV vaccine (FI-RSV) used in a clinical trial in the 1960s [4]. Possible factors involved in priming for ERD by non-replicating vaccines include disruption of protective epitopes by the chemical inactivation of the virus, poor innate receptor activation by the vaccine resulting in induction of poorly neutralizing antibodies and excess Th2-type responses [5,6]. Recent studies indicate that addition of Toll-like receptor (TLR) ligands, used as vaccine adjuvants, improve antibody affinity and Th1-skewing and prevent priming for ERD [7,8]. Furthermore, mucosal (i.e., intranasal, IN) immunization prevents induction of this complication and additionally induces secretory IgA (S-IgA) responses in the respiratory tract, which can act as a first line of defense against RSV [8,9]. Thus, new candidate RSV vaccines should induce Th1-skewed immune responses with induction of protective systemic and mucosal antibodies without priming for enhanced pathology upon natural infection. Mucosal vaccines that include TLR ligands for activation of innate receptors could be promising in this respect.

Virosomes are non-replicating virus-like particles consisting of reconstituted membranes of enveloped viruses [10]. The production of virosomes does not use chemicals (e.g. formalin) that could possibly modify protective epitopes. Upon production, virosomes allow the incorporation of lipophilic adjuvants, such as lipophilic TLR or NOD-like receptor (NLR)ligands, in their membranes. We previously reported on the feasibility of inclusion of lipophilic TLR ligand adjuvants (i.e. TLR2 ligand Pam$_3$CSK$_4$ and TLR4 ligand Monophosphoryl Lipid A; MPLA) in RSV virosomes and demonstrated that such adjuvant-supplemented virosomes have the capacity to induce protective antibodies after parenteral administration to mice or cotton rats, without priming for enhanced disease [11,12]. However, we have not yet investigated whether such virosomal RSV vaccine candidates are suited for use in mucosal immunization strategies.

TLR ligands have been reported to have the capacity to potentiate immune responses against mucosally delivered antigens [13]. In this respect, we found that a TLR9 ligand (i.e. CpG DNA), alone or co-formulated with a NOD2 ligand (i.e. L18-MDP), could boost mucosal and systemic antibody

responses to admixed inactivated RSV whole virions upon IN administration to mice [8]. However, lipophilic TLR ligands like Pam_3CSK_4 or MPLA can be much more efficiently incorporated in virosomes compared to CpG DNA [11,12]. The latter also would need additional incorporation of cationic lipids in the virosomal membrane in order to bind the negatively charged DNA molecules [14]. We therefore chose to explore the use of Pam_3CSK_4 as a TLR ligand adjuvant in virosomes. The TLR for Pam_3CSK_4, i.e. TLR2, is abundantly expressed on many cell types in mucosal tissues and does not need additional co-receptors like those described for the receptor of MPLA (TLR4), i.e. CD14 and MD2, that have low expression levels in mucosal tissues [15]. Also, other TLR2 ligands have shown promise as mucosal adjuvants [16-18].

In this study, we explored the use of the TLR2 ligand Pam_3CSK_4 in RSV-virosomes for potentiation of immune responses. We further investigated the use of a NOD2 ligand with possible mucosal immuno-adjuvant properties, i.e. L18-MDP [19,20], and its combined use with the TLR2 ligand, Pam_3CSK_4. The rationale for the combined use is that NOD2 ligands may synergistically enhance immune activation induced by TLR ligands [21-23], which would result in a better immunopotentiation by the mucosal virosomal RSV-vaccine. We demonstrate that incorporation of TLR2 ligands and/or NOD2 ligands in virosomes potentiates their capacity to activate a mouse macrophage cell line and human TLR/NOD2-expressing cells *in vitro*. *In vivo*, incorporation of a TLR2 ligand in virosomes boosted RSV-specific serum IgG and mucosal IgA responses after IN immunization of mice. While virosome incorporation of NOD2 ligand alone did not potentiate antibody responses, incorporation of NOD2 ligand in virosomes carrying a TLR2 ligand further stimulated local IgA and serum IgG responses. Adjuvantation of RSV virosomes with TLR2/NOD2 ligands also primed for a Th1-skewed response. Finally, RSV virosomes adjuvanted with TLR2/NOD2 ligands protected mice against challenge with infectious RSV without inducing enhanced disease.

2. Materials and Methods

2.1 Ethics statement

All animal experiments were evaluated and approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, University of Groningen, The Netherlands, according to the guidelines provided by the Dutch Animal Protection Act (permit number DEC 5239B). Immunizations and challenges were carried out under isoflurane anesthesia and every possible effort was made to minimize suffering of the animals.

2.2 Virus production and cell culture

RSV strain A2 (ATCC VR 1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in roller bottles on HEp-2 cells (ATCC,

CL-23, Wesel, Germany) 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 10% FBS (Lonza-Biowhittkar, Basel, Switzerland), and purified by a combination of differential and rate zonal ultracentrifugation on sucrose gradients. Purified virus was snap-frozen in liquid nitrogen and stored at –80°C in 20% sucrose in HNE buffer (5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7.4).

RAW-Blue (Mouse Macrophage Reporter Cell Line), HEK-Blue TLR2, HEK-Blue Null1, HEK-Blue NOD2, HEK-Blue Null2 cell lines, were purchased from Invivogen (Toulouse, France) and maintained according to the manufacturer's instructions. The abbreviation HEK stands for Human Embryonic Kidney.

2.3 Preparation of vaccine formulations

Virosomal RSV vaccine was produced as described earlier [11]. Briefly, purified virus was pelleted by ultracentrifugation for 30 min at 40, 000 rpm at 4°C, and the pellets were suspended in sterile HNE buffer. Then, this suspension was mixed with an equal volume of 200 mM 1,2-dicaproyl-sn-glycero-3-phosphocholine (DCPC) resulting in dissolution of the viral envelopes. The viral nucleocapsid was removed by ultracentrifugation at 50,000 rpm for 30 min at 4°C. Then, the supernatant containing the viral envelopes was added to a thin film of lipids prepared in a glass tube of 2:1 molar mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA) in 2:1 chloroform/methanol at 850 nmol/mg of viral envelop proteins. The lipid mixture was evaporated to dryness on the wall of a glass tube and traces of the solvents were removed at a high vacuum. The lipopeptide adjuvant,N-pamitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-(lysyl)3-lysine (Pam$_3$CSK$_4$, EMC Microcollections GmbH, Tubingen, Germany, lyophilized from the HCl solution), and/ or L-18 muramyldipeptide (L18-MDP) (6-O-stearoyl-N-Acetyl-muramyl-L-alanyl-D-isoglutamine; Invivogen, Toulouse, France) were dissolved in 100 mM DCPC in HNE, pH 7.4 and the solution was filtered through a 0.22 μm filter. To prepare virosomes, supernatant containing the viral envelopes and DCPC was combined with a thin film of lipid mixture, while to prepare adjuvanted virosomes, the lipopeptide solutions (Pam$_3$CSK$_4$) and/or L18-MDP were added separately or together (1 mg of adjuvant(s) per mg of viral protein). The mixture was incubated for 15 min at 4°C, filtered through 0.22 μm filter and dialyzed against 4x 2 liters of HNE buffer pH 7.4 in a sterile slide-A-lyzer (10 kD cut-off; ThermoScientific, Etten, Leur, the Netherlands) for 48 h. The buffer was changed 4 times. The virosomes were harvested and protein concentration was determined by Bio-Rad Bradford protein assay.

FI-RSV vaccine was prepared according to the protocol, which was used for the 1960s FI-RSV vaccine preparation as reported in [24]. This vaccine was diluted in HNE buffer to contain 5 μg of RSV protein in 25 μL.

2.4 In vitro analyses

The virosomal formulations were analyzed by equilibrium density gradient centrifugation on 10-60% sucrose gradients in HNE. The gradients were centrifuged for 60 h in an SW55 Ti rotor at 50000 rpm and the samples from the gradients were analyzed for protein, phospholipid and density (by refractometry). Later, each fraction was dialyzed against HNE in a slide-A-Lyzer MINI Dialysis device (Thermo Scientific, Geel, Belgium) overnight to remove the sucrose. Then, samples were corrected for increase in volume due to dialysis and 100 µL of the samples were used to stimulate each cell line i.e. Mouse Macrophage Reporter Cell Line (RAW-Blue cells) and Human Embryonic Kidney cell lines (HEK-Blue TLR2, HEK-Blue Null1, HEK-Blue NOD2, HEK-Blue Null2). RAW-Blue cells were used to measure vaccine/innate receptor ligand-induced NF-κB activation. These cells express all TLRs (except TLR5) as well as RIG-I, MDA5, NOD1 and NOD2 and carry a NF-κB responsive-gene encoding secreted alkaline phosphatase. RAW-Blue Cells ($1x10^5$ cells/well in 100 µL) were incubated with 100 µL sample overnight at 37°C in a 96-well flat bottom plates in triplicate. Alkaline phosphatase was quantified by incubating 20 µL cell supernatants with 180 µL Quanti-Blue (Invivogen, Toulouse, France) for 30 min at 37°C. Next, absorbance was measured at 630 nm through plate reader. Next, the relative amount of NF-κB induced by the gradient (virosomal) fractions was calculated by comparing to the NF-κB induced by CpG ODN, which was used as positive control. To study the stimulating capacity of the (virosomal) fractions to activate human TLR, HEK-Blue cells (HEK-TLR2, HEK-Null1, HEK-NOD2 and HEK-Null2; $5x\ 10^4$ cells/well) were incubated with 100 µL of the (virosomal) fractions in a 96-well flat bottom plate overnight at 37°C, 5% CO_2 atmosphere. Secreted alkaline phosphatase was assayed as indicated above. The relative amount of NF-κB induced in TLR2/Null1 and NOD2/Null2 cells was calculated by comparing to NF-κB induced by TNF-α (100 ng/mL) stimulation, used as positive control.

2.5 Immunization schedule and RSV challenge

Female specified-pathogen-free BALB/c OlaHsd mice (6-8 weeks old) purchased from Harlan, Zeist, The Netherlands, were used for all immunization experiments. Mice were immunized either with RSV virosomes (5 µg) alone or with incorporated innate receptor ligands, i.e. TLR2 (Pam_3CsK_4) and/ or NOD2 ligands (L18-MDP) present at a 1:1 weight ratio of ligand to vaccine antigen, respectively. Mice (6 mice per group) were immunized on days 0 and 21, under 3-4.5% isoflurane anesthesia in O_2 by IN inoculation of 50 µL. One group of mice was immunized with FI-RSV vaccine by intramuscular (IM) injection of 25 µL of FI-RSV absorbed to aluminium hydroxide (see above) and served as a control for vaccine-induced ERD. Another group of mice was immunized by IN inoculation with live-virus ($1x\ 10^6$ $TCID_{50}$) and served as a control for optimal anti-viral immunity. On day 28, all mice were challenged with live-virus ($1x\ 10^6$ $TCID_{50}$) by administration of 5x 10 µL of virus in the nose under isoflurane anesthesia.

2.6. Collection of blood samples and mucosal washes

Blood samples were drawn twice during the experiment: on day 28 before challenge by orbital puncture and day 32 by heart puncture. Sera were obtained after centrifugation of coagulated blood at 12,000 rpm for 10 min, and samples were stored at −20°C until further analysis. Bronchoalveolar lavages (BAL) and nasal washes were performed as previously described [25]. Briefly, lung lavages were performed by gentle injection of 1 mL PBS into the lungs with a syringe connected to the trachea, followed by subsequent aspiration of 1 mL of the wash fluid. Nasal washes were done by injection of 1 mL PBS retrograde via the trachea into the naso-pharynx and the lavage fluid was collected at the nostrils. The cellular components in the washes were removed by low-speed centrifugation. The supernatants were stored at −20°C until further analysis.

2.7 Antibody titer determination through ELISA

The antibody response to RSV was determined using enzyme-linked immunosorbent assay (ELISA). ELISA plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) were coated with beta-propiolactone (BPL) inactivated whole RSV (BPL-RSV) at 0.5 µg protein per well in coating buffer (0.05M carbonate–bicarbonate, pH 9.6–9.8) overnight at 37°C. Plates were washed three times with coating buffer and blocked with a 2.5% solution of milk powder (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) in coating buffer for 45 min at 37°C, then washed twice with coating buffer and three times with PBS Tween (PBST), containing 0.05% Tween 20 (Merck, Schiphol-Rijk, The Netherlands). Serial two-fold dilutions of serum samples (for IgG, IgG1 and Ig2a, IgA, IgE) and BAL and nasal wash samples (for IgA, IgG determination) were applied to the plates and incubated for 90 min. Plates were washed three times with PBST and incubated with a 1:5000 dilution of horseradish-peroxidase conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA; Southern Biotech, Birmingham, AL, USA) for 60 min at 37°C. Subsequently, the plates were washed three times with PBST and three times with PBS. After aspiration, *o*-Phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA) in 50 mM phosphate buffer pH 5.6 with 0.02% H_2O_2 was added and wells were incubated for 30 min. Then, the reaction was stopped by adding 50 µL 2M H_2SO_4 per well and the optical densities (OD) of the wells at 490 nm was determined. IgA levels were expressed as OD-values of undiluted samples. IgG levels were expressed as titers and defined as the reciprocal of the highest dilution that gave an OD value of at least 0.2.

2.8 IFN-γ and IL-5 detection in stimulated splenocyte supernatants

Four days after the virus challenge, mice were sacrificed and spleens were harvested separately in 15 mL tubes containing Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen, Breda, The Netherlands) supplemented with 1% Penicillin/Streptomycin and 0.1% beta-mercaptoethanol (Invitrogen, Breda, The Netherlands) and 10% FCS (Lonza-Biowhittaker, Basel, Switzerland).

Then, spleens were processed individually for *in vitro* stimulation. Briefly, washed spleens were passed through a 70 µm mesh (BD Biosciences, Heidelberg, Germany) using sterile 3 mL syringe plungers. Subsequently, erythrocytes were lysed by incubating with hypotonic medium (0.83% NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 5 min on ice. The cells were washed with IMDM, counted and brought to appropriate concentrations. Fresh spleen cells were seeded into 96-well plates at a concentration of 2x 10^6 cells/mL and stimulated with BPL-RSV (10 µg/mL) in IMDM/10% FCS in triplicates and incubated at 37°C in a 5% CO_2 atmosphere for 72 h. Supernatants were harvested and stored at –20°C until further analysis. IFN-γ and IL-5 cytokines were measured in supernatants of these stimulated splenocytes. For this, mouse IFN-γ- and mouse IL-5-high sensitivity ELISA kits (eBioscience, Vienna, Austria) were used according to the manufacturer's instruction. Detection limits were 15 pg/mL and 4 pg/mL for IFN-γ and IL-5, respectively.

2.9 Lung virus titration

Lungs were removed aseptically from all mice following euthanasia and washed in Dulbecco's Modified Essential Medium (DMEM), (PAA Laboratories, Colbe, Germany), supplemented with 2% FCS and transferred into 4 mL tubes containing 1 mL medium. Then, the lungs were homogenized individually with an automated Potter homogenizer Polytron-Aggregate® (Thomas Scientific, Swedesboro, NJ, USA), centrifuged at 1400 rpm for 10 min at 4°C and supernatants were separated. Virus titers were determined, by titration of the tissue-culture infectious dose ($TCID_{50}$). Briefly, a serial two fold dilutions of these samples were made in 96-well plates in quadruplicates with 1:5 starting dilution. HEp-2 cells, (20,000 per well) were seeded to the virus dilutions and incubated for 5 days at 37°C in a 5% CO_2 atmosphere. Then, supernatants were removed and plates were washed with PBS. The cells were then fixed with 1% para-formaldehyde in PBS for 1 h. After blocking cells with 2% milk powder (Protifar plus, Nutricia, Zoetermeer, The Netherlands) in PBS for 45 min at 37°C, plates were stained with 50 µL 1:400 dilution of FITC-labeled goat anti-RSV antibody (Meridian life science Inc, Saco, ME, USA) at 37°C overnight. The next day, plates were washed with PBS and analyzed under fluorescent microscope. Wells were considered positive for infection when ≥1 fluorescent syncytium was detected. Finally, $TCID_{50}$ titers were calculated by the Reed-Muench method using an Excel spreadsheet.

2.10 Lung histopathology

The lung lobes were harvested four days post infection, inflated with 4 % formalin in PBS for overnight and subsequently embedded in paraffin. Then, four µm slices were prepared, stained with standard hematoxylin and eosin (H & E) and were photographed using Nanozomer (Hamamatsu). Each lung section was analyzed for one of the following four parameters of pulmonary inflammatory changes: peribronchiolitis (inflammatory cells

surrounding a bronchiole), perivasculitis (inflammatory cells surrounding a small blood vessel), alveolitis (inflammatory cells within alveolar spaces), and interstitial pneumonitis (increased thickness of alveolar walls associated with inflammatory cells) by light microscopic analysis of slides.

2.11 Data analysis

All statistical analyses were performed using Graphpad Prism v5.0 (Graphpad Software, San Diego California, USA). Statistical significance was determined using unpaired Mann-Whitney U test. *P* values ≤0.05 were considered statistically significant.

3. Results

3.1 Characterization of virosomal formulations

Virosomal RSV formulations were prepared according to the protocol described in the Materials and Methods section. For all virosomal RSV-preparations, protein and phospholipids were found to co-migrate in the density gradients, indicating the successful formation of virosomes (Figure 1A). To investigate whether the lipophilic adjuvants were associated with the RSV virosomes, gradient fractions containing the virosomes, and top and bottom gradient fractions without virosomes (as controls), were tested for their capacity to induce NF-κB in TLR or NOD2 receptor-expressing mouse macrophage cell lines *in vitro*. Non-adjuvanted RSV virosome fractions poorly induced NF-κB expression in this assay (Figure 1B). Incorporation of Pam_3CSK_4 or L18-MDP in RSV virosomes clearly potentiated the capacity of the virosomes to induce NF-κB (Figure 1B). Incorporation of both ligands in virosomes enhanced NF-κB induction compared to the NF-κB induction by single incorporated ligands, although not in a synergistic fashion (Figure 1B). Top and bottom gradient fractions induced low NF-κB levels, suggesting that most of the added ligands was efficiently incorporated in the virosomal membranes.

Virosomal RSV formulations were also added to human cell lines that express single human innate receptors. Non-adjuvanted RSV virosomes again poorly induced NF-κB expression in TLR2 or NOD2-expressing cell lines (i.e. HEK-TLR2- or HEK-NOD2) cells, respectively (Figure 1C). RSV virosomes with incorporated Pam_3CSK_4 enhanced NF-κB expression in HEK-TLR2 cells, but not in HEK-NOD2 cells. Similarly, RSV virosomes with incorporated L18-MDP enhanced NF-κB expression in HEK-NOD2 cells, but not in HEK-TLR2 cells. Some residual bioactivity of L18-MDP was seen in the top fraction from the RSV-L18-MDP virosome density gradient, suggesting that most, but not all ligand was incorporated into the viral membranes. Since a large proportion of the lipophilic ligands was found to be incorporated into the viral membranes, we used non-fractionated virosomes for all subsequent immunization experiments.

3.2 In vivo immunogenicity

To evaluate the immunogenicity of the virosomal preparations upon mucosal administration, mice were immunized intranasally (IN) with RSV virosomes alone or RSV virosomes with incorporated Pam_3CSK_4 and/or L18-MDP. Control groups included non-immunized mice (HNE group), and mice immunized by live virus infection. One group of mice was immunized IM with FI-RSV vaccine to represent the mirror image of the FI-RSV vaccine used in 1960s clinical trial mentioned above.

Mice immunized with RSV virosomes with incorporated Pam_3CSK_4 showed significantly higher IgG antibody responses compared to responses induced by non-adjuvanted RSV virosomes (Figure 2A). Incorporation of

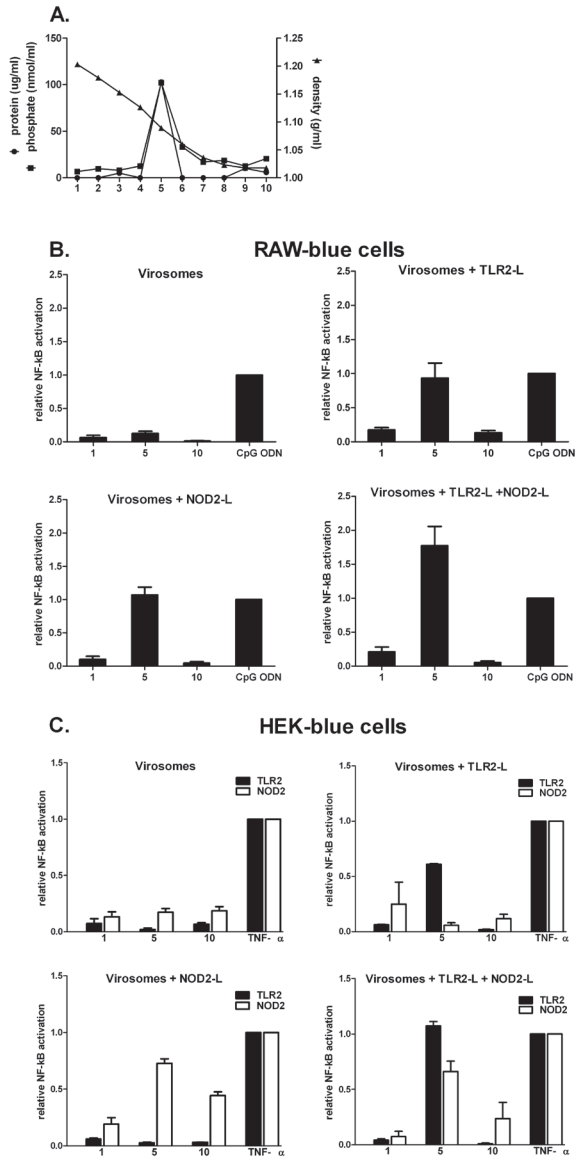

Figure 1. ***In vitro*** **analysis of RSV virosomes and RSV virosomes adjuvanted with TLR2 and /or NOD2 ligands.** RSV virosomes and RSV virosomes adjuvanted with TLR2 and/or NOD2 ligands were spun on an equilibrium density sucrose gradient. Subsequently, density, phospholipids and protein concentrations of each fraction was determined. Panel A shows a representative profile of a virosome purification gradient. Fractions (1,5,10; representing bottom, virosomal and top gradient fractions, respectively) were analyzed to determine their capacity to activate NF-κB in mouse macrophages (RAW-Blue cells; panel B) and human embryonic kidney cells (HEK-Blue TLR2 & HEK-Blue NOD2 cells; panel C). The level of NF-κB induced in RAW-blue cells was expressed as values relative to levels of NF-κB induced by CpG ODN, the positive control. To assess non-specific NF-κB activation by TLR2 and NOD2 ligand-carrying virosomes in HEK cells, control cells (HEK-Blue Null1 & HEK-Blue Null2 cells, respectively) were incubated with the same fractions and these values were subtracted from values obtained with HEK-Blue TLR2 & HEK-Blue NOD2 cells, respectively. As a control, HEK-Blue TLR2 and HEK-Blue NOD2 cells were stimulated with 100 ng/mL TNF-α. Bars represent the NF-κB activation relative to TNF-α control.

L18-MDP in virosomes also induced increased IgG antibody responses. Moreover, additional incorporation of L18-MDP into the Pam_3CSK_4-containing virosomes further enhanced IgG antibody responses.

In order to analyze the phenotype of the immune responses, Th1-signature IgG2a and Th2-signature IgG1 subtype antibodies were determined. Incorporation of Pam_3CSK_4 alone or combined with L18-MDP into RSV virosomes resulted in a significant increase of IgG2a/IgG1 ratios after IN-immunization of mice. Incorporation of L18-MDP alone did not result in an increase of IgG2a/IgG1 ratios. As expected, live RSV induced higher IgG2a/IgG1 ratios than FI-RSV (Figure 2B). Thus, incorporation of TLR2/NOD2 ligands in IN-administered RSV virosomes significantly stimulates systemic RSV-specific IgG antibody responses with a more pronounced production of Th1-signature IgG2a antibodies.

In order to determine mucosal immune responses, nasal washes and BAL samples were taken for analysis of IgA and IgG antibody levels. We found a significant induction of nasal and BAL RSV-specific IgA antibodies in mice immunized IN with RSV virosomes with incorporated Pam_3CSK_4 or live virus (Figure 3A,C). Incorporation of L18-MDP in the Pam_3CSK_4-virosomes further boosted mucosal IgA antibody responses (Figure 3A,C). We also determined

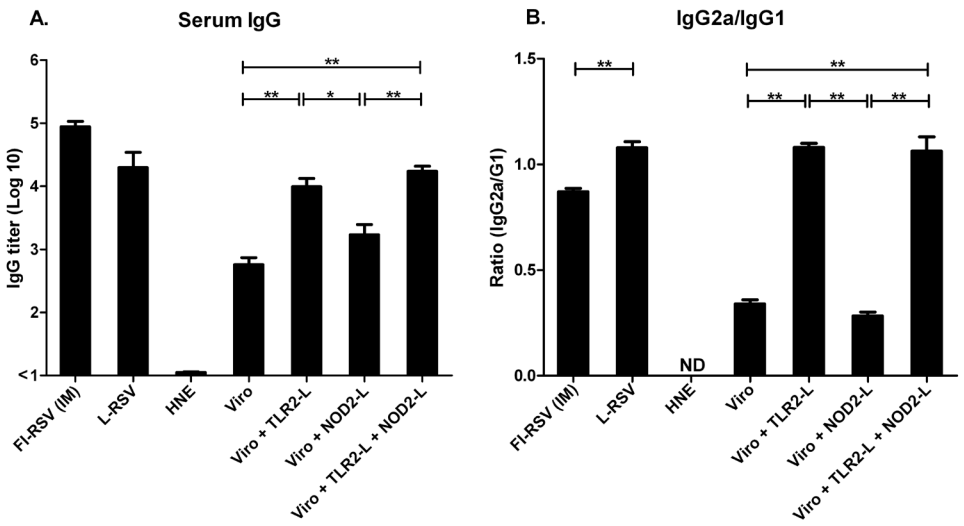

Figure 2. RSV-specific systemic IgG antibody responses after IN immunization of mice. BALB/c mice were immunized IN with RSV virosomal vaccine formulations (5 µg of protein) or HNE. Control mouse groups were either immunized IM with FI-RSV or IN with live-RSV (L-RSV) on day 0 and 21. One week after the booster immunization, RSV-specific IgG responses in serum (A) and IgG-subtypes (IgG2a/IgG1) (B) were determined by ELISA. Panel A: Bars represent the geometric mean titer and standard deviation. Panel B: Bars represent the ratios of IgG2a/IgG1. The data shown are representative of at least 3 separate experiments. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

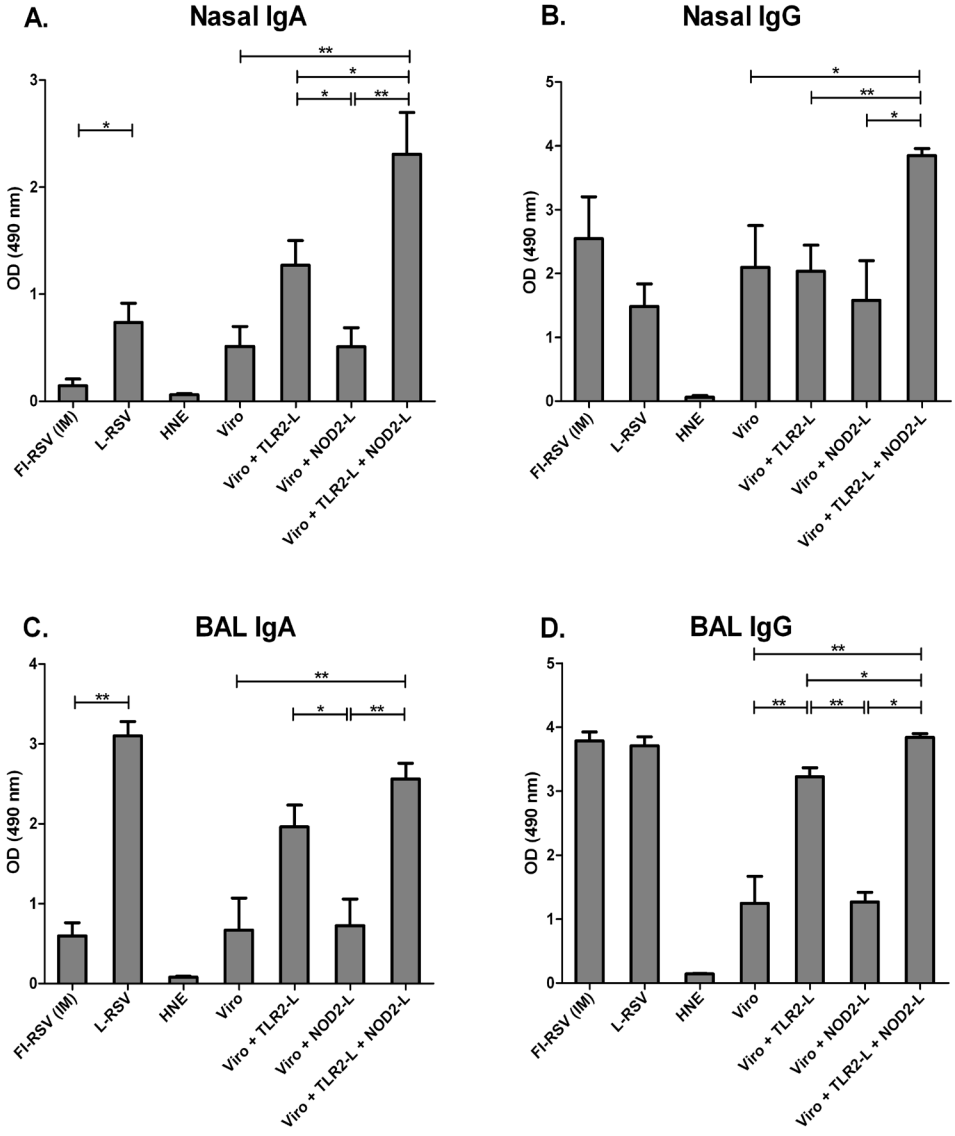

Figure 3. RSV-specific mucosal IgA and IgG antibody responses in nasal washes and BAL after IN immunization of mice. BALB/c mice were immunized IN with RSV virosomal vaccine formulations (5 µg of protein) or HNE. Control mouse groups were either immunized IM with FI-RSV or IN with L-RSV on day 0 and 21. Four days after challenge with live RSV (day 32), RSV-specific IgA responses in nasal washes (A), BAL (C) were determined by ELISA. RSV-specific IgG responses in nasal washes (B) and BAL (D) were also determined. Panels A-D: Bars represent the mean absorbance (490 nm) and standard deviation. The data shown are representative data of at least 3 separate experiments. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

IgG antibody levels in nasal washes and BAL. Nasal RSV-specific IgG was observed in all immunized groups, but the highest levels were seen in the groups immunized IN with virosomes adjuvanted with both ligands (Figure 3B). Immunization with adjuvanted virosomes induced BAL IgG to similar levels as FI-RSV or live virus immunization. Again, additional incorporation of L18-MDP in Pam_3CSK_4-adjuvanted virosomes further boosted IgG levels (Figure 3D).

To characterize the humoral immune response in more detail, we analyzed RSV-specific serum IgA and IgE antibody levels. Significant levels of RSV-specific serum IgA were induced after immunization with live virus and RSV-virosomes containing Pam_3CSK_4, but not after immunization with FI-RSV or virosomes with L18-MDP. Induction of serum IgE antibodies, a hallmark of atopic responses, was only observed after immunization with FI-RSV. Thus, RSV-virosomes with incorporated TLR2/NOD2 ligands induce local as well as serum RSV-specific IgA and IgG antibody responses upon mucosal immunization and no IgE antibody responses.

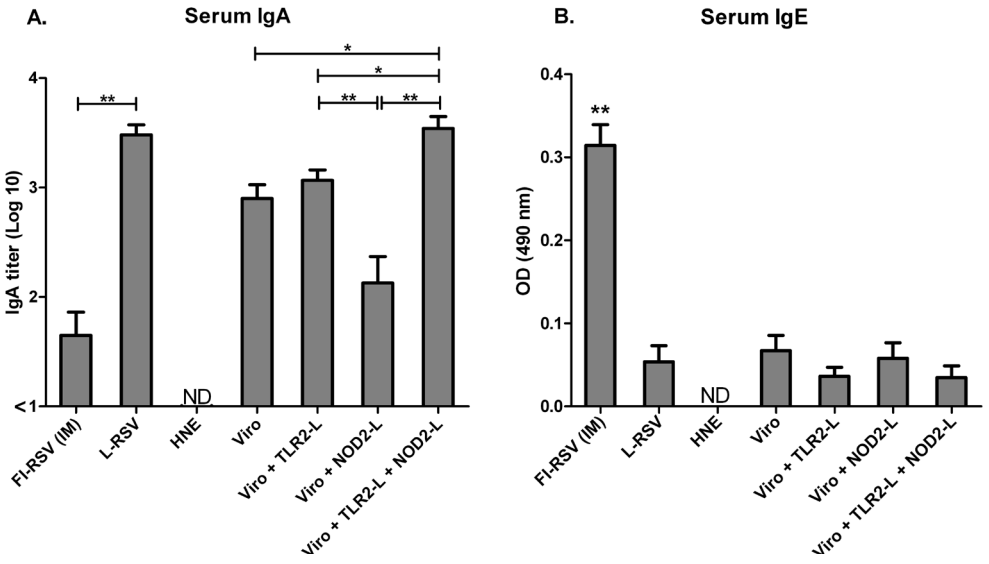

Figure 4. RSV-specific serum IgA and IgE antibody responses. BALB/c mice were immunized IN with RSV-virosomal vaccine formulations (5 µg of protein) or HNE. Control mouse groups were either immunized IM with FI-RSV or IN with L-RSV on day 0 and 21. RSV-specific serum IgA (A) and IgE (B) were determined by ELISA. Panal A: Bars represent the geometric mean titer and standard deviation. Panel B: Bars represent the mean absorbance (490 nm) and standard deviation. The data shown are representative data of at least 2 separate experiments. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

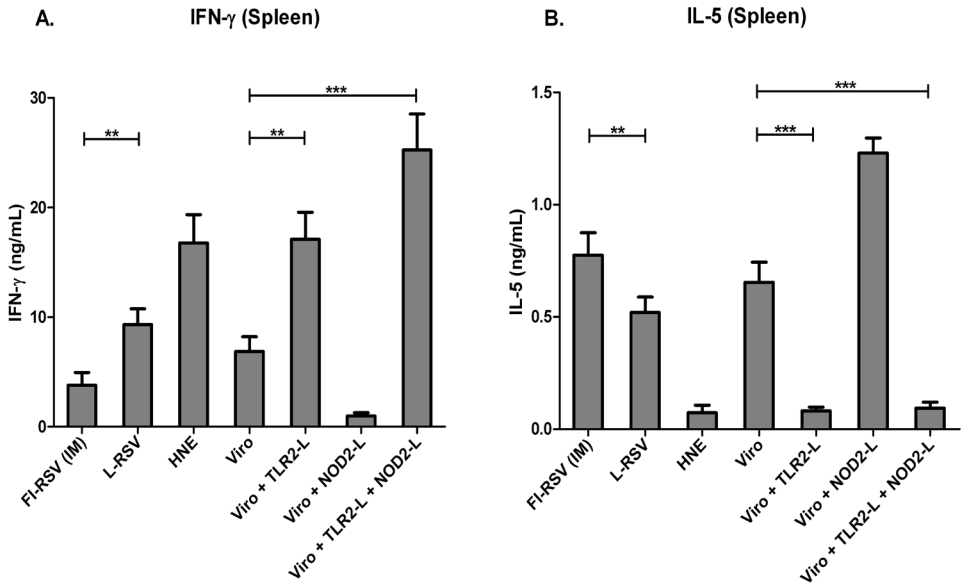

Figure 5. *Ex vivo* cytokine production by splenocytes in response to stimulation with RSV. IFN-γ (Panel A) and IL-5 (Panel B) production in splenocyte cultures after stimulation with inactivated RSV was determined by cytokine ELISA. Cytokines in the culture supernatants were assayed after 3 days of culturing. Bars and error bars represent means ± SD. The data shown are representative of at least 3 separate experiments. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference.
* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

3.3 RSV-specific cell-mediated immune responses

As excess Th2-skewed T cell responses may contribute to ERD, we investigated whether the RSV-specific T cell responses had Th1-/or Th2-skewed phenotypes. To this end, we analyzed IFN-γ and IL-5 levels in *ex vivo* RSV-restimulated splenocytes from immunized and subsequently challenged mice of all groups. Incorporation of Pam$_3$CSK$_4$ in virosomes induced Th1-skewing: it significantly increased IFN-γ responses while IL-5 responses were significantly reduced (Figure 5). Clear-cut Th2-skewed responses, indicated

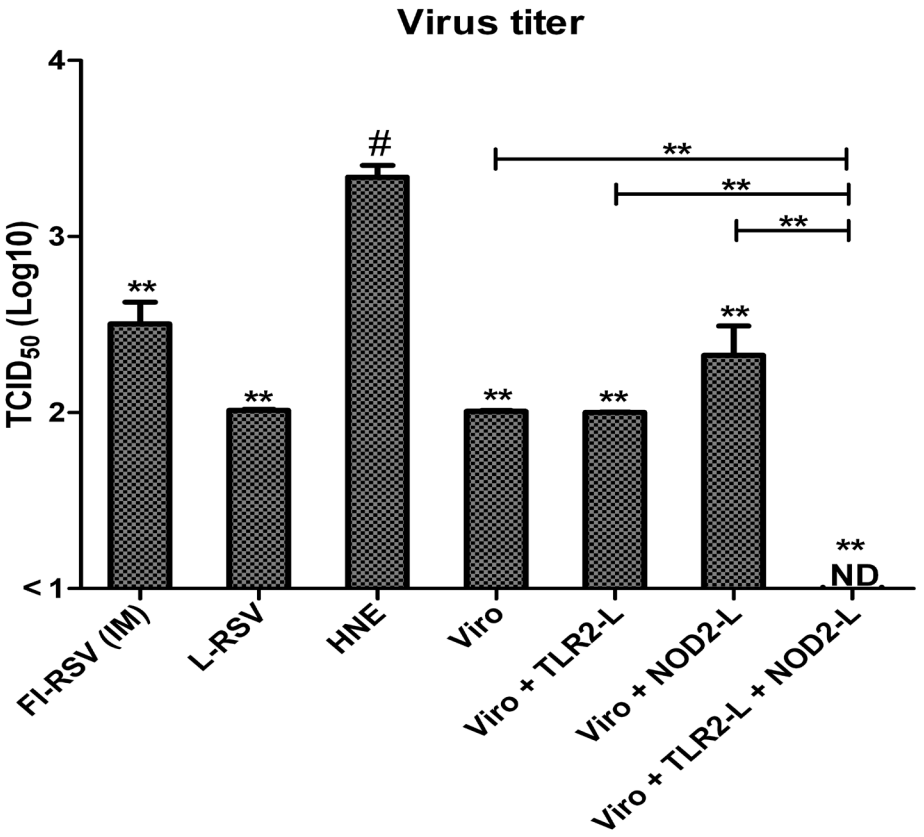

Figure 6. Protection of mice from challenge with live RSV. BALB/c mice were immunized IN with RSV-virosomal vaccine formulations (5 µg of protein) or HNE. Control mouse groups were either immunized IM with FI-RSV or IN with L-RSV on day 0 and 21. Mice were challenged with live-RSV on day 28 and four days after challenge (day 32), lung viral titers were determined. Viral titers are expressed as $TCID_{50}$. Bars and error bars represent means ± SD. The data shown are representative of at least 2 separate experiments. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

by high IL-5 but low IFN-γ responses, were induced by FI-RSV and by L18-MDP-adjuvanted RSV virosomes (Figure 5). Incorporation of L18-MDP in Pam$_3$CSK$_4$-adjuvanted virosomes did not lead to Th2-skewing, but appeared to further increase the Th1-skewing by enhancing IFN-γ secretion (Figure 5). This, however, did not reach a statistically significant difference. Thus, RSV virosomes supplemented with TLR2/NOD2 ligands efficiently prime for safe Th1-phenotype responses upon mucosal immunization in mice.

3.4 Protection from live RSV challenge

In addition to immune parameters, protection against viral challenge was investigated. For this, immunized mice were infected by IN inoculation of live virus, one week after the booster immunization. Four days later, lung viral titers were measured. All immunized mice showed significantly reduced viral titers compared to viral titers seen in non-immunized mice. Only mice immunized with RSV-virosomes with both ligands incorporated had undetectable viral titers (Figure 6).

3.5. Lung pathology

To examine possible occurrence of ERD upon challenge of immunized mice, lungs were collected four days post-challenge virus and lung slices were examined. Mice immunized with FI-RSV showed clear signs of ERD, i.e. alveolitis and infiltration of cells in peribronchial and perivascular regions (Figure 7C), while non-immunized mice or mice immunized with live virus did not show any signs of ERD (Figure 7A, B). Lungs from mice immunized with non-adjuvanted virosomes, or virosomes with Pam$_3$CSK$_4$ and/or L18-MDP, did not show signs of ERD either (Figure 7D-G), although some areas with minor infiltration were observed (Figure 7E, F). Thus, unlike IM injection with FI-RSV, IN immunization with (adjuvanted) RSV virosomes does not prime for ERD.

4. Discussion

Mucosal delivery of vaccines has been explored as a non-invasive and highly acceptable route of administration and can induce mucosal antibody responses, in addition to systemic antibody responses. Since RSV enters through the respiratory mucosal site, mucosal immunity at these sites would contribute to prevention of infection [26,27]. However, non-replicating virus vaccines administered through the mucosal route generally induce poor immune responses. This poor immunogenicity may, however, be overcome by co-administeration of mucosal adjuvants with the vaccine [13,28-30]. TLR2 ligands, like MALP-2 (macrophage-activating lipopeptide-2) and zymosan, for example, have been reported to have good mucosal immunoadjuvant properties [16,17]. Here, we show that the TLR2 ligand Pam$_3$CSK$_4$, incorporated in RSV virosomes, also has mucosal immuno-adjuvant

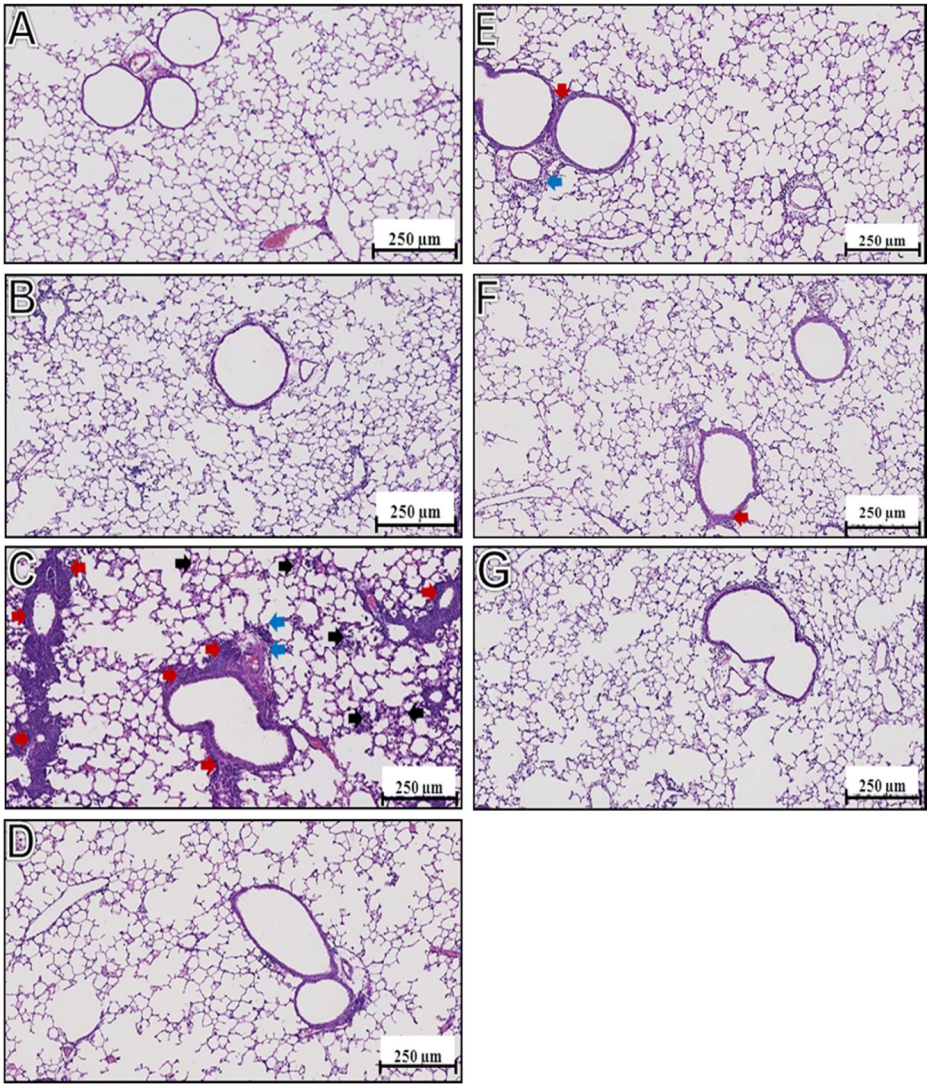

Figure 7. Immunopathology after challenge. BALB/c mice were immunized as described above. One week after the booster immunization, mice were challenged with liveRSV (1x 10^6 $TCID_{50}$). Four days after challenge, one lobe of lung was harvested, sliced and stained with H&E for pathology analysis using light microscopy. Panels are representative pictures of the lungs of mice immunized with (A) HNE, (B) L-RSV, (C) FI-RSV, (D) RSV virosomes, (E) RSV virosomes + TLR2-L, (F) RSV virosomes + NOD2-L and (G) RSV virosomes + TLR2-L + NOD2- L immunized mice. Black arrows indicate alveolitis, red arrows indicate peribronchiolitis and blue arrows indicate perivasculitis.

properties. No clearcut *in vivo* immunoadjuvant activity was observed when the NOD2 ligand L18-MDP was incorporated in virosomes. However, when it was incorporated in virosomes carrying TLR2 ligand, a further increase in *in vivo* antibody responses and Th1-skewing was observed. This points to a synergistic activity of the ligands in immunopotentiation, leading to increased RSV-specific immunity upon IN administration of the virosomal RSV vaccine. From the above data we conclude that Pam$_3$CSK$_4$, alone or in combination with L18-MDP, shows promise for use as a mucosal adjuvant in a non-replicating virosomal RSV vaccine.

We have previously shown that mucosal immunization with inactivated RSV, supplemented with TLR9 (CpG DNA) and NOD2 (L18-MDP) ligands, is an effective approach for induction of RSV-specific antibodies and Th1-skewed T cell responses [8]. In this study, we investigated the virosome platform as a candidate RSV vaccine, and chose to include the TLR2 ligand Pam$_3$CSK$_4$, alone or together with L18-MDP. Pam$_3$CSK$_4$ is a synthetic triacylated lipopeptide that, unlike CpG DNA for example, readily associates with virosomes by partitioning into the virosomal membrane during the reconstitution process (Figure 1) [11]. When incorporated in RSV virosomes, Pam$_3$CSK$_4$ enhances RSV-specific serum IgG and Th1 responses upon intramuscular immunization [11]. One way by which it potentiates immune responses is through induction of proinflammatory cytokines, which is initiated after binding to a heterodimeric TLR2/1 receptor and engagement of the MyD88-mediated signaling pathway [31]. A recent study showed that Pam$_3$CSK$_4$ not only upregulates pro-inflammatory genes, but also genes involved in leukocyte transendothelial migration at the site of vaccine administration [32]. Another possible factor contributing to the adjuvant activity of Pam$_3$CSK$_4$ could be its cationic nature. This property has been shown to enhance binding and uptake of RSV viral particles by target cells [33]. In a similar fashion, it could enhance binding and uptake of RSV virosomes that contain Pam$_3$CSK$_4$ by, for example, antigen-presenting cells. Besides this, a number of other activities of TLR2 ligands have been described that could contribute to enhancement of mucosal responses. These include the induction of increased antigen uptake by M cells [34,35], induction of T cell-independent B-cell activation and maturation leading to enhanced antibody secretion [16], and enhancement of IgA secretion by B cells [18]. Thus, TLR2 ligands, including Pam$_3$CSK$_4$, seem highly suited for immunopotentiation of vaccine-induced systemic and mucosal immune responses upon mucosal administration.

Although the NOD2 ligand L18-MDP has been reported to have mucosal immunoadjuvant activity [19], we observed no enhancement of mucosal antibody responses by virosome-incorporated L18-MDP upon IN immunization of mice. We did, however, observe a strong Th2-skewing by L18-MDP (Figure 5), a feature which has been described before [36]. Both FI-RSV and L18-MDP-adjuvanted virosomes induced Th2-skewed T cell responses, but the latter did not prime for ERD (Figure 7F). Notably, in contrast to FI-RSV, L18-MDP-adjuvanted virosomes do not induce RSV-specific IgE antibodies (Figure 4B), despite the strongly Th2-skewed responses. IgE is an important mediator of hypersensitivity responses including ERD and this

may be why mice immunized with L18-MDP virosomes did not show ERD. The lack of IgE induction may be explained by the active suppression of IgE responses by MDP or its derivatives upon mucosal administration [37]. Thus our data suggest that, on their own, Th2 responses do not readily cause ERD in mice but when associated with RSV-IgE responses contribute to ERD.

L18-MDP did not display Th2-skewing properties when combined with TLR ligands, such as CpG DNA [8] or Pam_3CSK_4 (Figure 5). Rather, L18-MDP enhances the TLR ligand-mediated activation leading to more pronounced RSV-specific IFN-γ secretion by splenocytes and significantly increased mucosal antibody responses. These data are in line with other studies showing that ligands for NOD-like receptors (NLR), such as NOD2, enhance TLR ligand-induced activation. For example, the TLR ligand induced activation, proliferation and survival of B cells was further enhanced by addition of NOD ligands [23]. Also, TLR ligand induced (Th1-skewing) cytokines in dendritic cells are further enhanced by supplementing with NOD1/NOD2 ligands [21]. Thus, RSV-specific serum and mucosal antibody responses and Th1 responses boosted by Pam_3CSK_4 can further be increased by addition of the NOD2 ligand L18-MDP.

As the respiratory tract is the port of entry for RSV, mucosal antibodies in the respiratory tract could significantly contribute to protection. It is likely that mucosal antibodies are important in protection of the upper respiratory tract, while serum antibodies mainly protect the lungs, as has previously been demonstrated for influenza infection [38]. In support of this notion, studies on RSV infection in adult humans and the elderly have shown that nasal antibodies are a better correlate of protection against RSV infection than serum antibodies [39]. On the other hand, high RSV-specific serum IgG has been shown to correlate with reduced disease severity upon RSV infection [40], which points to a role of serum IgG in protection of the lungs. In conclusion, IN-immunization with RSV virosomes with incorporated Pam_3CSK_4 alone, or combined with the NOD2 ligand L18-MDP is a promising strategy to induce RSV-specific immunity that includes serum and mucosal antibody responses and safe Th1-skewed cellular immune responses, without priming for enhanced disease.

5. References

1. Van Drunen Littel-van den Hurk,S., Mapletoft JW, Arsic N, Kovacs-Nolan J. (2007) Immunopathology of RSV infection: prospects for developing vaccines without this complication. Rev Med Virol 17(1): 5-34.
2. Dowell SF, Anderson LJ, Gary HE,Jr., Erdman DD, Plouffe JF, File TM,Jr. et al. (1996) Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults. J Infect Dis 174(3): 456-62.
3. Hall CB, Walsh EE, Long CE, Schnabel KC. (1991) Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 163(4): 693-8.
4. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K et al. (1969) Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 89(4): 422-34.
5. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. (1996) 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 70: 2852-60.
6. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP et al. (2009) Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med 15: 34-41.
7. Johnson TR, Rao S, Seder RA, Chen M, Graham BS. (2009) TLR9 agonist, but not TLR7/8, functions as an adjuvant to diminish FI-RSV vaccine-enhanced disease, while either agonist used as therapy during primary RSV infection increases disease severity. Vaccine 27: 3045-52.
8. Shafique M, Wilschut J, de Haan A. (2012) Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands. Vaccine 30(3): 597-606.
9. Hussell T, Humphreys IR. (2002) Nasal vaccination induces protective immunity without immunopathology. Clin Exp Immunol 130(3): 359-62.
10. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM et al. (2005) The virosome concept for influenza vaccines. Vaccine 23 (Suppl 1): S26-38.
11. Stegmann T, Kamphuis T, Meijerhof T, Goud E, de HA, Wilschut J. (2010) Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. Vaccine 28: 5543-50.
12. Kamphuis T, Meijerhof T, Stegmann T, Lederhofer J, Wilschut J, de Haan A. (2012) Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A in mice. PLoS One 7(5): e36812.
13. Lawson LB, Norton EB, Clements JD. (2011) Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. Curr Opin Immunol 23(3): 414-20.
14. De Jonge J, Leenhouts JM, Holtrop M, Schoen P, Scherrer P, Cullis PR et al. (2007) Cellular gene transfer mediated by influenza virosomes with encapsulated plasmid DNA. Biochem J 405(1): 41-9.
15. Parker D, Prince A. (2011) Innate immunity in the respiratory epithelium. Am J Respir Cell Mol Biol 45(2): 189-201.
16. Borsutzky S, Kretschmer K, Becker PD, Muhlradt PF, Kirschning CJ, Weiss S et al. (2005) The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. J Immunol 174(10): 6308-13.
17. Ainai A, Ichinohe T, Tamura S, Kurata T, Sata T, Tashiro M et al. (2010) Zymosan enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine. J Med Virol 82(3): 476-84.
18. Liang Y, Hasturk H, Elliot J, Noronha A, Liu X, Wetzler LM et al. (2011) Toll-like receptor 2 induces mucosal homing receptor expression and IgA production by human B cells. Clin Immunol 138(1): 33-40.
19. Fukushima A, Yoo YC, Yoshimatsu K, Matsuzawa K, Tamura M, Tono-oka S et al. (1996) Effect of MDP-Lys(L18) as a mucosal immunoadjuvant on protection of mucosal infections

by Sendai virus and rotavirus. Vaccine 14: 485-91.
20. Ogawa T, Shimauchi H, Hamada S. (1989) Mucosal and systemic immune responses in BALB/c mice to Bacteroides gingivalis fimbriae administered orally. Infect Immun 57(11): 3466-71.
21. Tada H, Aiba S, Shibata K, Ohteki T, Takada H. (2005) Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. Infect Immun 73(12): 7967-76.
22. Uehara A, Yang S, Fujimoto Y, Fukase K, Kusumoto S, Shibata K et al. (2005) Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. Cell Microbiol 7(1): 53-61.
23. Petterson T, Jendholm J, Mansson A, Bjartell A, Riesbeck K, Cardell LO. (2011) Effects of NOD-like receptors in human B lymphocytes and crosstalk between NOD1/NOD2 and Toll-like receptors. J Leukoc Biol 89(2): 177-87.
24. Prince GA, Denamur F, Deschamps M, Garcon N, Prieels JP, Slaoui M et al. (2001) Monophosphoryl lipid A adjuvant reverses a principal histologic parameter of formalin-inactivated respiratory syncytial virus vaccine-induced disease. Vaccine 19: 2048-54.
25. De Haan A, Geerligs HJ, Huchshorn JP, van Scharrenburg GJ, Palache AM, Wilschut J. (1995) Mucosal immunoadjuvant activity of liposomes: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes. Vaccine 13: 155-62.
26. Ogra PL, Faden H, Welliver RC. (2001) Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 14(2): 430-45.
27. Baca-Estrada ME, Foldvari M, Babiuk SL, Babiuk LA. (2000) Vaccine delivery: lipid-based delivery systems. J Biotechnol 83(1-2): 91-104.
28. Holmgren J, Lycke N, Czerkinsky C. (1993) Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. Vaccine 11(12): 1179-84.
29. Yamamoto S, Takeda Y, Yamamoto M, Kurazono H, Imaoka K, Yamamoto M et al. (1997) Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J Exp Med 185(7): 1203-10.
30. Roberts M, Bacon A, Rappuoli R, Pizza M, Cropley I, Douce G et al. (1995) A mutant pertussis toxin molecule that lacks ADP-ribosyltransferase activity, PT-9K/129G, is an effective mucosal adjuvant for intranasally delivered proteins. Infect Immun 63(6): 2100-8.
31. Hennessy EJ, Parker AE, O'Neill LA. (2010) Targeting Toll-like receptors: emerging therapeutics? Nat Rev Drug Discov 9(4): 293-307.
32. Caproni E, Tritto E, Cortese M, Muzzi A, Mosca F, Monaci E et al. (2012) MF59 and Pam3CSK4 boost adaptive responses to influenza subunit vaccine through an IFN type I-independent mechanism of action. J Immunol 188(7): 3088-98.
33. Nguyen DT, de Witte L, Ludlow M, Yuksel S, Wiesmuller KH, Geijtenbeek TB et al. (2010) The synthetic bacterial lipopeptide Pam3CSK4 modulates respiratory syncytial virus infection independent of TLR activation. PLoS Pathog 6(8): e1001049.
34. Cashman SB, Morgan JG. (2009) Transcriptional analysis of Toll-like receptors expression in M cells. Mol Immunol 47(2-3): 365-72.
35. Chabot SM, Chernin TS, Shawi M, Wagner J, Farrant S, Burt DS et al. (2007) TLR2 activation by proteosomes promotes uptake of particulate vaccines at mucosal surfaces. Vaccine 25(29): 5348-58.
36. Magalhaes JG, Fritz JH, Le Bourhis L, Sellge G, Travassos LH, Selvanantham T et al. (2008) Nod2-dependent Th2 polarization of antigen-specific immunity. J Immunol 181(11): 7925-35.
37. Auci DL, Kleiner GI, Chice SM, Athanassiades TJ, Dukor P, Durkin HG. (1998) Control of IgE responses. V. Oral administration of a synthetic derivative of the inner bacterial cell wall (SDZ 280.636) to sensitized mice induces isotype specific suppression of peak hapten specific IgE antibody forming cell responses, serum IgE levels and immediate hypersensitivity responses. Immunol Invest 27(1-2): 105-20.
38. Renegar KB, Small PA,Jr, Boykins LG, Wright PF. (2004) Role of IgA versus IgG in the

control of influenza viral infection in the murine respiratory tract. J Immunol 173(3): 1978-86.
39. Walsh EE, Falsey AR. (2004) Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. J Infect Dis 190(2): 373-8.
40. Walsh EE, Peterson DR, Falsey AR. (2004) Risk factors for severe respiratory syncytial virus infection in elderly persons. J Infect Dis 189(2): 233-8.

Chapter 4

An intranasal virosomal vaccine against Respiratory Syncytial Virus, supplemented with TLR2 and NOD2 ligands, induces RSV-specific systemic and mucosal mmunity in cotton rats

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Manuscript in preparation

Abstract

Respiratory syncytial virus (RSV) causes severe respiratory disease in infants and the elderly. There is no licensed RSV vaccine available. A mucosal RSV vaccine would be an attractive candidate as it could induce both systemic and mucosal antibody responses and does not readily prime for enhanced respiratory disease (ERD), as did the 1960s formalin-inactivated RSV vaccine (FI-RSV). Earlier, we found that an intranasal (IN) vaccine consisting of RSV virosomes with incorporated innate receptor ligand adjuvants had the capacity to induce both systemic and mucosal antibody responses in mice. Here, we investigated whether such an approach would induce protective immunity in cotton rats, an animal more permissive for RSV infection than mice. To this end, we produced non-adjuvanted RSV virosomes, RSV virosomes carrying Pam_3CSK_4, a TLR2 ligand, and RSV virosomes carrying Pam_3CSK_4 together with L18-MDP, a NOD2 ligand. The capacity of these preparations to induce protective systemic (serum IgG) and mucosal (S-IgA and IgG) antibody responses upon IN immunization in cotton rats was investigated. Also, we assessed whether these preparations primed for ERD, through analysis of immunopathology in the lungs after live virus challenge of immunized animals. All responses were compared with responses induced by IM immunization with alum-adjuvanted FI-RSV or IN immunization with live virus. We found that incorporation of Pam_3CSK_4 in RSV virosomes potentiates systemic IgG and lung S-IgA antibody responses capable of inhibiting viral replication in cotton rat lungs. Inclusion of L18-MDP in virosomes, carrying Pam_3CSK_4, further potentiated these responses. Immunization with live virus, but not IN immunization with adjuvanted virosomes or IM immunization with FI-RSV, induced nasal S-IgA. All immunized groups showed reduced viral lung titers upon challenge. Immunization with FI-RSV, but not IN immunization with adjuvanted virosomes or live virus, induced immunopathology (ERD). We conclude that mucosal immunization with RSV virosomes with incorporated TLR2 and/or NOD2 ligands is a promising approach to induce RSV-specific immunity in a safe fashion.

1. Introduction

Respiratory syncytial virus (RSV) infection causes viral bronchiolitis in infants and young children but also forms a serious risk for the elderly and for immunocompromised individuals [1-3]. RSV infection does not provide life-long protection and multiple reinfections can occur throughout the life of an individual. Despite the burden of RSV disease, there is still no registered vaccine available. This is in part due to the disastrous outcome of a clinical trial in 1960s using formalin-inactivated, alum-adjuvanted, whole RSV vaccine (FI-RSV) [4], which has considerably slowed down vaccine development. In this trial, young vaccinees developed enhanced respiratory disease (ERD) upon subsequent natural exposure to RSV and two of them died [4,5]. Recent progress in the understanding of the mechanisms underlying vaccine-induced ERD, however, has revived the development of inactivated RSV vaccine candidates [6].

Although many vaccines are administered through IM injection, the development of an RSV vaccine for mucosal administration, including intranasal (IN) administration, remains of great interest. IN administration forms an attractive alternative to IM injection, because it is an accessible, non-invasive and highly acceptable route of administration [7]. Clear benefits are that, in addition to the induction of systemic antibody responses, mucosal vaccine delivery potentially induces local secretory-IgA (S-IgA) responses that further aid in protection against respiratory viral infections, including RSV infection [8,9]. Also, mucosal immunization does not readily prime for enhanced disease in animal models, underlining the importance to explore this route for future vaccination strategies against RSV [9]. However, to break mucosal immune tolerance mechanisms, suitable mucosal adjuvants are likely to be needed in such an approach. In this respect, we previously showed, in a murine model system, that Toll-like receptors (TLR) ligands and NOD-like receptor ligands, admixed with inactivated whole RSV particles or incorporated in RSV virosomes, hold promise as adjuvants in mucosal RSV vaccine candidates [8].

Although mice are the most commonly used animal models for assessment of vaccine immunogenicity and efficacy, they are not very permissive for RSV replication. Cotton rats (*Sigmodon hispidus*) are approximately 100-fold more permissive to RSV infection than mice and can also mimic FI-RSV-induced immunopathology as seen in humans [10-12]. This model is, therefore, highly suitable for evaluation of the immunogenicity, safety and protective capacity of an intranasal virosomal RSV vaccine adjuvanted with TLR and NOD2 ligands.

Here we demonstrate that incorporation of a TLR2 ligand (Pam$_3$CSK$_4$) in RSV virosomes boosts RSV-specific serum IgG and mucosal IgA responses after IN immunization of cotton rats. Incorporation of a NOD2 ligand (L18-MDP) in RSV virosomes carrying TLR2 ligand further enhanced local IgA and serum IgG antibody responses. Finally, RSV virosomes supplemented with TLR2 and NOD2 ligands protected cotton rats against challenge with infectious RSV without inducing ERD. Taken together, these results show

that IN immunization of cotton rats with a virosomal RSV vaccine containing incorporated TLR2 and NOD2 ligands represents a safe and efficacious potential RSV vaccine candidate.

2. Materials and Methods

2.1 Ethical statement

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

2.2 Virus and cell culture

RSV strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in roller bottles on HEp-2 cells (ATCC, CL-23, Wesel, Germany) 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 gradient as described before [8].

2.3 Vaccine formulations

Virosomal RSV vaccine was produced as described earlier [13]. Briefly, purified virus was dissolved in 100 mM 1,2-dicaproyl-sn-glycero-3-phosphocholine (DCPC) in HNE buffer (5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7.4) and the viral nucleocapsid was removed by ultracentrifugation. Then, a 2:1 molar mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA) in 2:1 chloroform/methanol at 850 nmol/mg protein was evaporated to a dry film in a glass tube and traces of the solvents were removed at a high vaccuum. The lipopeptide adjuvant,N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-(lysyl)3-lysine (Pam_3CSK_4, EMC Microcollections GmbH, Tubingen, Germany, lyophilized from the HCl solution), and/ or L18 muramyldipeptide (L18-MDP) (6-O-stearoyl-N-Acetyl-muramyl-L-alanyl-D-isoglutamine; Invivogen, Toulouse, France) was dissolved in 100 mM DCPC in HNE buffer and the solution was filtered through a 0.22 µm filter. To prepare virosomes, viral DCPC supernatant was combined with a thin film of dry lipid mixture in glass tube and, in case of adjuvanted-virosomal formulations, the lipopeptide solution (Pam_3CSK_4) alone or Pam_3CSK_4 and L18-MDP-adjuvants together (1 mg of adjuvant(s) per mg of viral protein) was added. The mixture was incubated for 15 min at 4°C, filtered through a

0.22 µm filter and dialyzed against HNE buffer in a sterile slide-A-lyzer (10 kD cut-off; Thermo Scientific, Etten, Leur, the Netherlands) for 48 h. The virosomes were harvested and the protein concentration was determined by a Bio-Rad Bradford protein assay.

FI-RSV was prepared as reported elsewhere [12]. This vaccine was diluted in HNE buffer to contain 5 µg of RSV protein in a 50 µL volume.

2.4 Cotton rat immunizations and RSV challenge

Female outbred cotton rats (Hsd: Cotton Rat) of 4-6 weeks old were purchased from Harlan (Indianapolis, IN, USA). Cotton rats (6 animals per group) were immunized IN with 100 µL of RSV-virosomes (5 µg protein) alone or with incorporated innate receptor ligands, i.e. TLR2 (Pam$_3$CSK$_4$) and NOD2 ligands (L18-MDP) at approximately a 1:1 ratio of ligand to vaccine antigen, respectively. Control rats received 100 µL of live virus (1x 10^6 $TCID_{50}$) or HNE or 50 µL (5 µg viral proteins) of FI-RSV IM. Cotton rats were immunized on days 0 and 21, under 3-4.5% isoflurane anesthesia in O_2. On day 28, animals were challenged with 1x 10^6 $TCID_{50}$ RSV IN. At the time of second immunization and challenge, blood was drawn by retro-orbital puncture in order to analyze post-prime and post-booster immunization antibody levels. Animals were sacrificed four days after the challenge and lung washes (bronchoalveolar lavages; BAL) were performed as described before [8]. 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 analysis.

2.5 Immunological assays

RSV-specific antibody titers were determined using enzyme-linked immunosorbent assay (ELISA) as described earlier [8]. Briefly, 96-well ELISA plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) were coated with beta-propiolactone (BPL) inactivated whole RSV (BPL-RSV) at 0.5 µg protein per well in coating buffer (0.05M carbonate–bicarbonate, pH 9.6–9.8) overnight at 37°C. Plates were washed three times with coating buffer and blocked with a 2.5% solution of milk powder (Protifar Plus, Nutricia, Zoetermeer, The Netherlands) in coating buffer. Plates were incubated for 90 min with two-fold serial dilutions of sera or nasal/lung washes starting at dilutions of 1:200 for serum or 1:1 dilutions of washes. After washing, plates were incubated with a 1:5000 dilution of horseradish-peroxidase conjugated goat anti-mouse IgG, or IgA; Southern Biotech, Birmingham, AL, USA) which bind to both mouse and cotton rat IgG and IgA, respectively. Subsequently, the plates were stained with *o*-Phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA) in 50 mM phosphate buffer pH 5.6 with 0.02% H_2O_2 for 30 min. Then, the reaction was stopped by adding 2M H_2SO_4 and absorption was measured at 490 nm.

2.6 Virus titration and micro-neutralization assay

Virus titers were determined by $TCID_{50}$ as described before [14]. Briefly, lungs were removed aseptically after euthanasia of the animals. Lungs were then homogenized in 1 mL of 2% FBS containing HEp-2 medium using an automated Potter homogenizer Polytron-Aggregate® (Thomas Scientific, Swedesboro, NJ, USA). Next, homogenates were centrifuged at 1400 rpm for 10 min at 4°C, and supernatants, diluted to a 1:8 starting dilution, were used to determine viral titers using the $TCID_{50}$ method as previously described [14].

For determination of RSV virus-neutralization titers, sera were decomplemented by heat inactivation for 30 min at 56°C. Then, serum neutralization was evaluated by incubating different dilutions of decomplemented sera with 70 $TCID_{50}$ of RSV for 2 h and addition of this mixture to HEp-2 cells in 96-well plates. Following 5 day incubation, wells positive for RSV infection were scored and the neutralizing titer was calculated [14]. The neutralization titer was calculated by using the Reed & Muench method for determination of the 50% end point titer.

2.7 Lung histopathology

The inflated cotton rat lungs were embedded in paraffin and 4 µm slices were prepared. Then, the slides were stained with hematoxylin and eosin (H & E) using standard protocols and photographed using NanoZoomer (Hamamatsu Photonics, Hamamatsu city, Japan). Each lung section was analyzed for one of the following four parameters of pulmonary inflammatory changes: peribronchiolitis (inflammatory cells surrounding a bronchiole), perivasculitis (inflammatory cells surrounding a small blood vessel) and alveolitis (inflammatory cells within alveolar spaces) by light microscopic analysis of slides.

2.8 Data analysis

Statistical analyses were performed using Graphpad Prism v5.0 (Graphpad Software, San Diego California, USA). Statistical significance was determined using unpaired Mann-Whitney U test. *P* values ≤ 0.05 were considered to represent statistically significant differences.

3. Results

3.1 Immunogenicity in cotton rats

To evaluate the immunogenicity of the virosomal RSV vaccine upon mucosal administration, cotton rats were immunized IN with RSV virosomes alone or RSV virosomes with incorporated Pam$_3$CSK$_4$, or RSV virosomes with incorporated Pam$_3$CSK$_4$/L18-MDP adjuvants. Control groups included cotton rats immunized IN with buffer ("HNE" group), immunized by live virus infection, or immunized IM with a FI-RSV adsorbed to alum. A priming IN immunization with (adjuvanted) virosomal RSV vaccine induced only low levels of serum IgG, but levels could be increased by giving a booster immunization (Figure 1). IN immunization with RSV virosomes containing both adjuvants induced significantly higher serum IgG levels compared to IN immunization with non-adjuvanted virosomes or virosomes containing Pam$_3$CSK$_4$ alone (Figure 1). Both IM immunization with FI-RSV or live virus infection induced high levels of RSV-specific serum IgG which were not increased by giving a booster immunization (Figure 1).

In order to determine mucosal immune responses, we analyzed lung wash samples for analysis of RSV-specific IgA and IgG antibody levels. We found a significant induction of RSV-specific IgA antibodies in cotton rats immunized IN with live virus or RSV virosomes with incorporated adjuvant(s) (Pam$_3$CSK$_4$ alone or combined with L18-MDP; Figure 2). We also determined IgG antibody levels in lung washes. RSV-specific IgG was detected in animals immunized with FI-RSV, live virus and adjuvanted RSV virosomes and levels reflected serum IgG levels (Figure 2). In addition to lung wash antibody levels, antibody levels were also determined in nasal washes. RSV-specific nasal IgA was detected in cotton rats that received live virus, but not in other groups. Nasal wash IgG was detected in cotton rats immunized with FI-RSV and cotton rats that received live virus immunization. Thus, IN immunization with RSV virosomes containing innate receptor ligands induces RSV-specific serum IgG and mucosal IgA and IgG antibodies, the latter mainly in the lung.

3.2 Virus neutralization

Next, the virus-neutralization capacity of the sera was evaluated. Sera from non-immune cotton rats (HNE group) did not show any neutralizing activity, while sera from cotton rats that received live virus infection showed the highest level of neutralization, which was significantly higher than the level observed in FI-RSV-immunized cotton rats. Inclusion of adjuvant in IN-administered RSV virosomes significantly stimulated the mean neutralizing titer of the immune sera (Figure 3). However, a number of animals that received adjuvanted RSV virosomes IN did not show neutralizing capacity in their sera (Figure 3), probably due to a too low concentration of RSV-specific IgG in the serum.

3.3 Protection from live RSV challenge

In addition to immune parameters, protection against viral infection was measured. For this, immunized cotton rats were infected by IN inoculation of live virus, one week after the booster immunization. Four days later, lung viral titers were measured. All immunized cotton rats showed significant reduction of viral titers compared to viral titers seen in non-immune cotton rats (HNE group; Figure 4). Cotton rats immunized with RSV virosomes adjuvanted with Pam_3CSK_4 had significantly reduced viral titers in their lungs when compared to titers seen in non-immune animals. Cotton rats immunized IN with RSV virosomes containing both ligands, live virus or FI-RSV had undetectable viral titers (Figure 4).

3.4 Lung immunopathology upon RSV infection

To evaluate the safety of IN administration of RSV virosomes adjuvanted with Pam_3CSK_4 and/or L18-MDP adjuvants, we harvested the lungs of immunized and challenged cotton rats and assessed lung histology. The lungs of non-immune animals did not show signs of enhanced disease, i.e. no symptoms of alveollitis, and the peribronchial and perivascular regions were free of cell infiltrates (Figure 5A,B). In contrast, lungs from cotton rats immunized IM with FI-RSV showed prominent alveollitis as well as peribronchial and perivascular infiltrates (Figure 5E,F). On the other hand, the lungs of the animals immunized IN with RSV virosomes alone (Figure 5G,H), RSV virosomes adjuvanted with Pam_3CSK_4 (Figure 5I,J), RSV virosomes with incorporated Pam_3CSK_4 and L18-MDP adjuvants (Figure 5K,L), or live virus (Figure 5C,D) did not show any signs of ERD.

4. Discussion

Mucosal administration of vaccines has been recognized as an attractive route for vaccine administration and can induce mucosal in addition to systemic antibody responses. Non-replicating virus vaccines, like RSV virosomes, administered through mucosal routes generally are not very immunogenic. To potentiate immune responses, we have incorporated lipophilic TLR2 and NOD2 ligands in the RSV virosomal membrane. In order to study whether the immune responses induced by IN immunization are capable of inhibiting pulmonary RSV replication and are safe with respect to the occurrence of ERD, we here used the cotton rat model. Cotton rats are much more permissive to RSV infection than mice and can mimic the characteristic FI-RSV-induced immunopathology seen in humans [10-12]. We demonstrate that incorporation of Pam_3CSK_4 and L18-MDP in RSV virosomes potentiates systemic IgG and respiratory tract S-IgA antibody responses capable of inhibiting viral replication in cotton rat lungs without priming for enhanced disease.

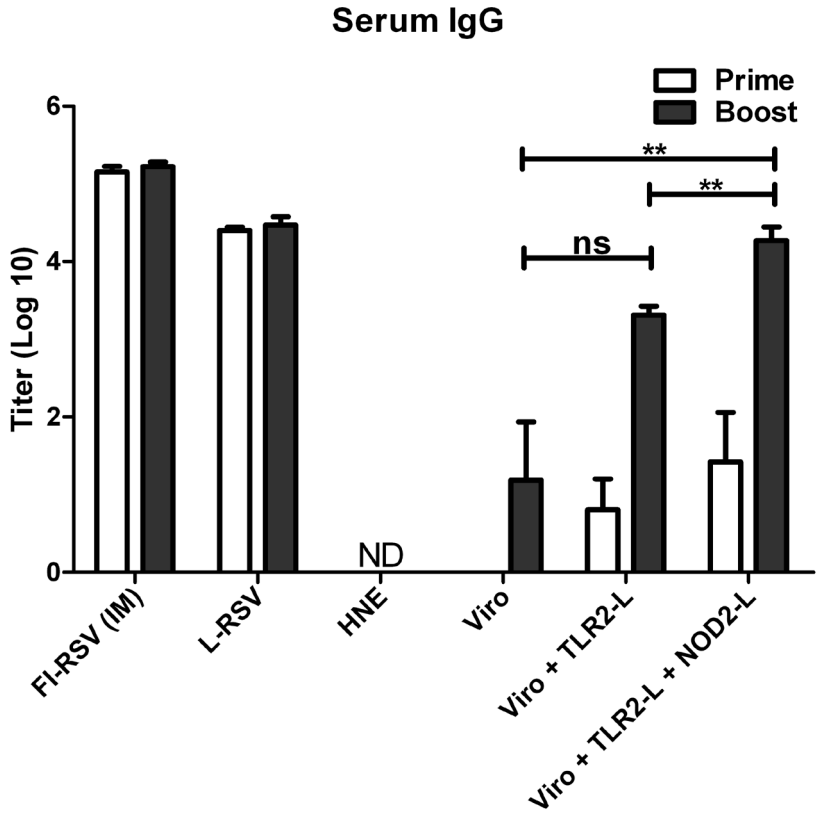

Figure 1. RSV-specific systemic IgG antibody responses in cotton rats. Cotton rats were immunized IN with virosomal RSV vaccine formulations (5 µg of protein) or HNE. Control groups were either immunized IM with FI-RSV or IN with live RSV (L-RSV). Immunizations were done on day 0 and 21 (primary and booster immunization, respectively). Blood was drawn on day 21 and one week after the booster immunization. RSV-specific IgG antibody responses were determined by ELISA. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

RSV targets mucosal surfaces of the respiratory tract and protection against infection depends on the presence of virus-neutralizing antibodies. Previous studies in humans have shown that the presence of RSV-specific serum antibodies mainly correlates with protection against symptomatic disease and that the levels negatively correlate with severity of disease in risk groups such as the elderly [15]. Furthermore, Walsh *et al*. found that low nasal IgA levels correlate with a higher risk to develop RSV infection [16]. Our data show that IN immunization with RSV virosomes, supplemented with built-in innate receptor ligands, induces serum IgG that contributes to protection of the lungs. It is likely that the lung S-IgA induced by the vaccine contributes to protection as well. For example, sera from a number of animals that received virosomes adjuvanted with Pam$_3$CSK$_4$/L18-MDP had

undetectable virus-neutralization activity but, yet, these animals did not show viral shedding in their lungs. This could mean that lung S-IgA aids in the protection as well. Nasal S-IgA was not detected after IN immunization with RSV virosomes, supplemented with innate receptor ligands, but an efficient S-IgA response was induced by IN immunization with live virus (Figure 2). In preliminary experiments, we found that cotton rats immunized with live

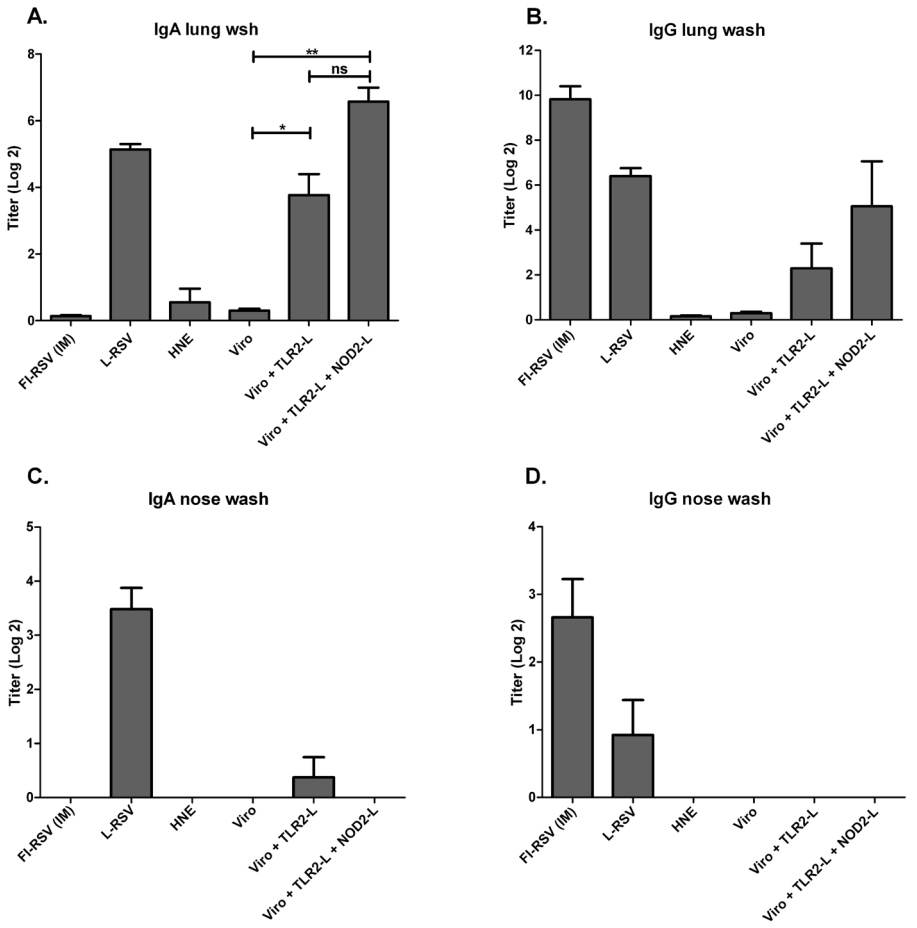

Figure 2. RSV-specific mucosal IgA and IgG antibody in lung and nasal washes. Cotton rats were immunized IN with virosomal RSV vaccine formulations (5 µg of protein) or HNE. Control groups were either immunized IM with FI-RSV or IN with live RSV (L-RSV). Immunizations were done on day 0 and 21 (primary and booster immunization, respectively). Four days after challenge with live RSV (day 32), RSV-specific IgA the lung washes (A), nasal washes (C) were determined by ELISA. RSV-specific IgG in lung washes (B) and nasal washes (D) were also determined. Panels A-D: Bars represent the mean titers (2 log) and standard deviation. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

virus did not show virus shedding in the nasal tissue while other immunized groups usually did show nasal viral shedding, although at very variable levels (data not shown). Although this suggests that S-IgA inhibits virus infection in the nasal tissue, it cannot be excluded that nasal IgG contributes to protection as well. Our data suggest that some IgG transudates to the nasal tissue when serum IgG levels are high. For example, IM injection of alum-adjuvanted FI-RSV for example, leads to high RSV-specific serum IgG and the appearance of these antibodies at the nasal mucosa (Figure 1, 2). It remains to be further investigated if a lack of nasal S-IgA reduces local protection against RSV, like in humans [16].

Whereas, in the present study in cotton rats, we did not find nasal S-IgA induction upon IN immunization with adjuvanted RSV virosomes, it is of interest to note that, in mice, the RSV virosomes adjuvanted with TLR2/NOD2 ligands induced higher nasal S-IgA responses upon IN immunization than responses induced by live virus infection (Chapter 3). The relative lack of stimulatory activity of TLR ligand adjuvants on nasal S-IgA antibody responses in the cotton rat was also observed when we tested an IN virosomal RSV vaccine adjuvanted with MPLA (a TLR4 ligand) in mice or cotton rats (Chapter 5). Why RSV virosomes, supplemented

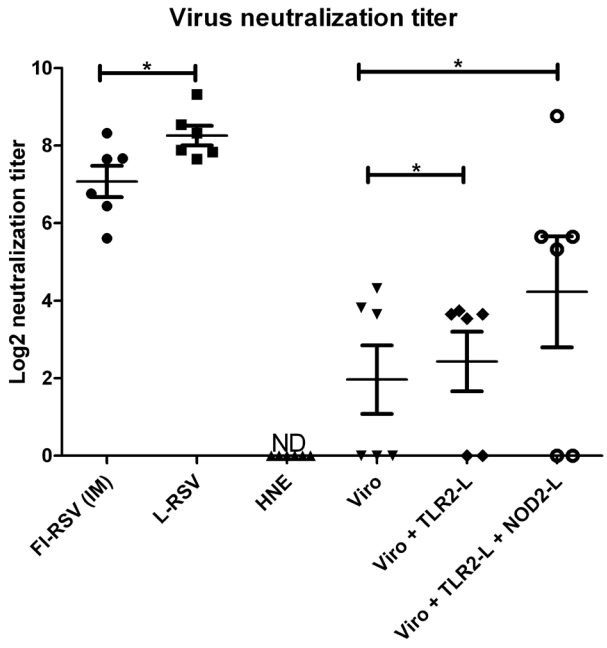

Figure 3. Virus neutralization in cotton rats. Cotton rats were immunized as described in Figure 1. Virus-neutralizing antibody titers were determined in serum samples taken at day 28. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$

with innate receptor ligands, efficiently induce nasal S-IgA in mice, but not in cotton rats, remains unclear. A possible explanation could be that the dose of antigen and/or adjuvant given to the cotton rats was too low. In our studies, a similar dose of antigen and adjuvant (i.e. 5 µg of protein and 5 µg of the respective adjuvants) was given to mice and cotton rats, while cotton rats weigh approximately 10 times more than mice. In studies employing IN immunization of cotton rats, Prince *et al.* used a lower dose of RSV antigen (up to 1.25 µg), yet a much higher dose of adjuvant (20-100 µg of the TLR9 ligand CpG DNA) for induction of full protective antibody responses [17]. In comparison, we previously found that a 4 µg dose of CpG DNA, co-administered IN with inactivated RSV, could potentiate nasal S-IgA responses in mice. Therefore, increased doses of innate receptor ligand adjuvants could possibly potentiate nasal S-IgA responses in cotton rats.

In conclusion, our data demonstrate that an IN administered virosomal RSV vaccine adjuvanted with TLR2/NOD2 ligands induces serum IgG along with lung IgA as well as lung IgG antibodies in cotton rats. This response provides protection against challenge with infectious virus and, importantly, does not lead to enhanced immunopathology in the lungs upon infection of the animals with RSV. The IN virosomal vaccine, adjuvanted with TLR2/NOD2 ligands, therefore holds promise for further development as a potential RSV vaccine.

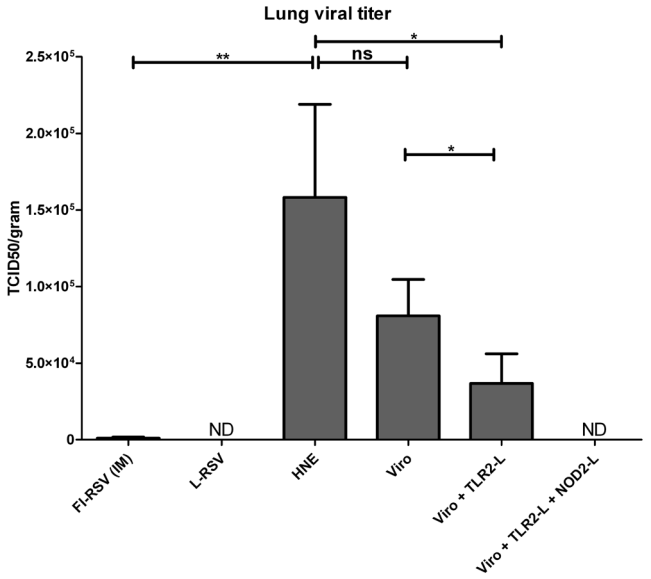

Figure 4. Protection in cotton rats from challenge with live RSV. Cotton rats were immunized IN with virosomal RSV vaccine formulations (5 µg of protein) or HNE. Control mouse groups were either immunized IM with FI-RSV or IN with live RSV (L-RSV). Immunizations were done on day 0 and 21 (primary and booster immunization, respectively). Cotton rats were challenged with live RSV on day 28 and four days after challenge (day 32) cotton rats were sacrificed and lungs were harvested and viral titers were determined. Viral titers are expressed as $TCID_{50}$. Bars and error bars represent means ± SD. Data was analyzed by a Mann-Whitney U test and a *p*-value of ≤ 0.05 was considered to represent a significant difference. * $p \leq 0.05$, ** $p \leq 0.01$.

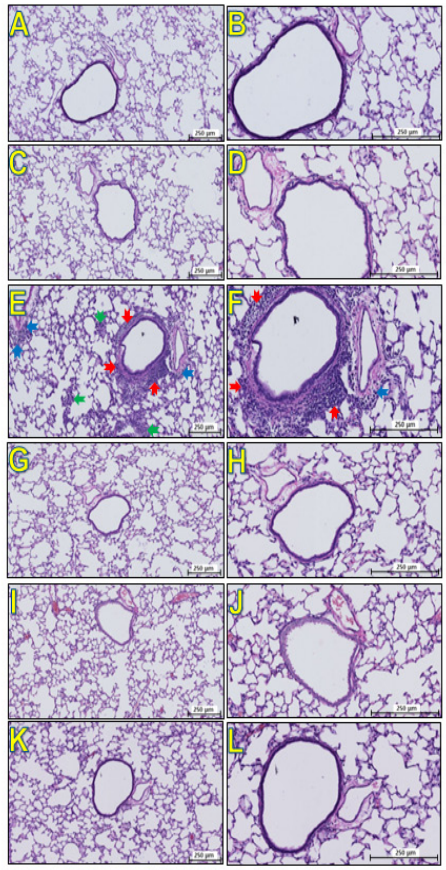

Figure 5: Lung pathology in cotton rats. Cotton rats were immunized, challenged and terminated as in Figure 4. After termination, one lung lobe was fixated with 4% formalin under 20 cm water pressure to retain the structure of the lung. After fixation, the lungs were embedded in paraffin and 4 µm slices were cut and stained with H&E. The lungs were evaluated by light microscopy. Panels are representative pictures of the lungs of immunized group. Groups: HNE (A,B); live virus (C,D); FI-RSV (E,F); RSV virosomes (G,H); RSV virosomes + Pam$_3$CSK$_4$ (I,J); RSV virosomes + Pam$_3$CSK$_4$ + L18-MDP (K,L). Red arrows: peribronchial infiltrates, green arrows: alveolar infiltrates and Blue arrows: perivascular infiltrates.

5. References

1. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin Microbiol Rev 2010;23(1):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(17):1749-59.
3. Raboni SM, Nogueira MB, Tsuchiya LR, Takahashi GA, Pereira LA, Pasquini R et al. Respiratory tract viral infections in bone marrow transplant patients. Transplantation 2003;76(1):142-6.
4. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969;89(4):422-34.
5. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am J Epidemiol 1969;89(4):405-21.
6. van Drunen Littel-van den Hurk,S., Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. Rev Med Virol 2007;17(1):5-34.
7. Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev 2006;58(1):68-89.
8. 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(3):597-606.
9. Hussell T, Humphreys IR. Nasal vaccination induces protective immunity without immunopathology. Clin Exp Immunol 2002;130(3):359-62.
10. Boukhvalova MS, Prince GA, Blanco JC. The cotton rat model of respiratory viral infections. Biologicals 2009;37(3):152-9.
11. Prince GA, Jenson AB, Hemming VG, Murphy BR, Walsh EE, Horswood RL et al. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactiva ted virus. J Virol 1986;57(3):721-8.
12. 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(Pt 12):2881-8.
13. Stegmann T, Kamphuis T, Meijerhof T, Goud E, de HA, Wilschut J. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. Vaccine 2010;28(0264-410; 34):5543-50.
14. 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(5):e36812.
15. Walsh EE, Peterson DR, Falsey AR. Risk factors for severe respiratory syncytial virus infection in elderly persons. J Infect Dis 2004;189(2):233-8.
16. Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. J Infect Dis 2004;190(2):373-8.
17. Prince GA, Mond JJ, Porter DD, Yim KC, Lan SJ, Klinman DM. Immunoprotective activity and safety of a respiratory syncytial virus vaccine: mucosal delivery of fusion glycoprotein with a CpG oligodeoxynucleotide adjuvant. J Virol 2003;77(24):13156-60.

Chapter 5

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 S-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 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 dicaproyl-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, St Louis, MO, USA). After 30 min the staining was stopped by addition of 2M H_2SO_4 and absorption was measured at 492 nm. IFN-γ 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 (Figure 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 developed IgG antibodies, to similar levels as in mice that received a second immunization with FI-RSV or a live virus infection (Figure 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 (Figure 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 (Figure 1D).

3.2 Cellular immune response in mice

Next, we determined Th1-type cytokine (IFN-γ) and Th2-type cytokine (IL-5) levels in RSV-restimulated splenocytes from immunized and subsequently challenged animals of all groups (Figure 2). IFN-γ 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 (Figure 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 (Figure 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 (Figure 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.

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 (Figure 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 (Figure 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 (Figure 5A).

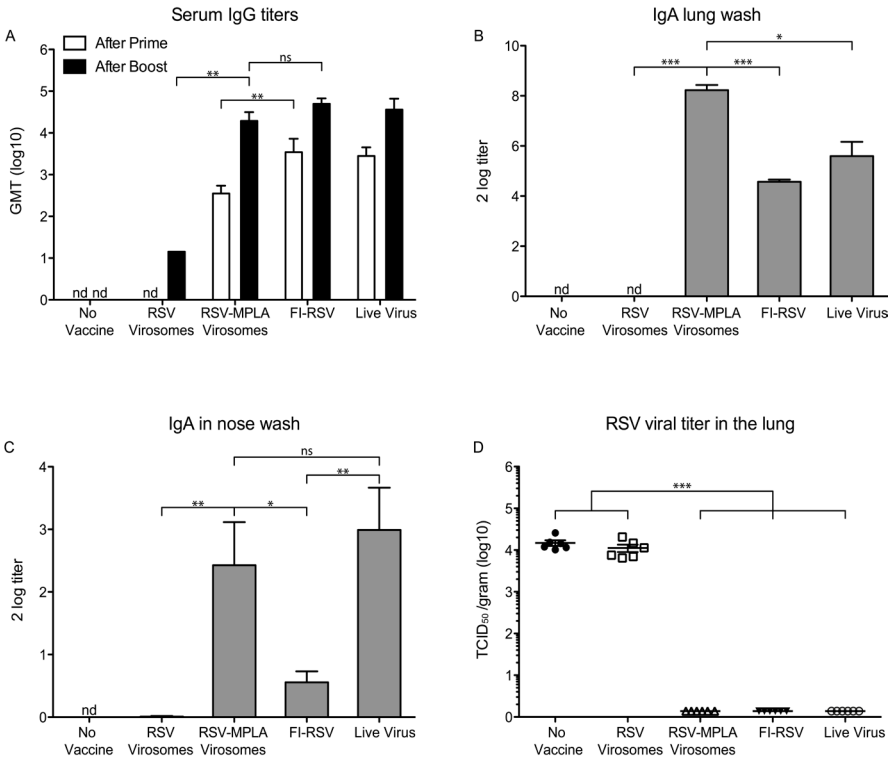

Figure 1. Immunogenicity and protection in mice. Mice were immunized twice ("prime" on day 0 and "boost" on day 14) with RSV virosomes IN, RSV-MPLA virosomes IN, FI-RSV IM, and live virus IN. Control mice received buffer IN. Fourteen days after the immunizations blood was drawn and RSV-specific IgG in 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).

3.5 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 (Figure 6C,D). The lungs of non-immunized animals (Figure 6A,B), animals immunized IN with RSV virosomes (Figure 6E,F), RSV-MPLA virosomes (Figure 6G,H) or live virus (Figure 6I,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 (Figure 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.

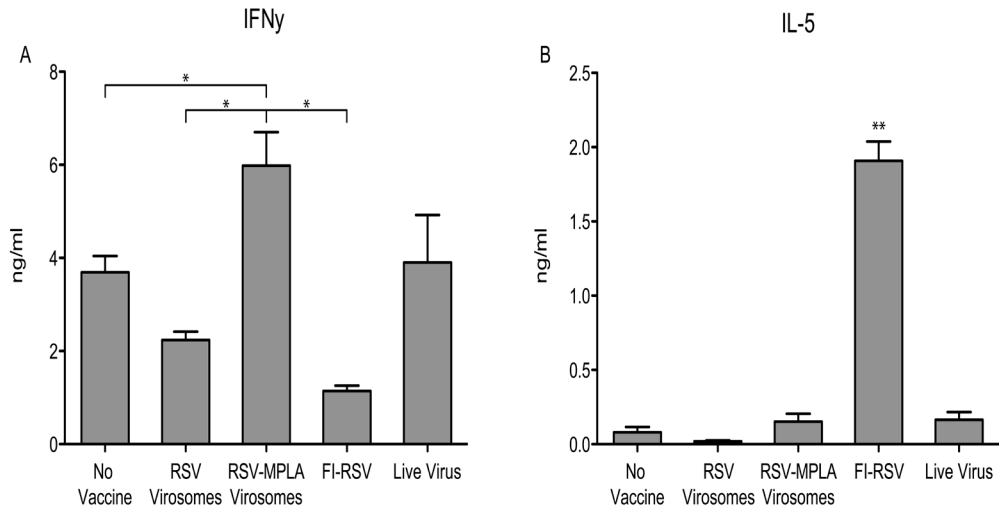

Figure 2. Cellular immune response in mice. Mice were immunized, challenged and terminated as in Figure 1. After termination, spleens were harvested and splenocytes were restimulated *in vitro* with BPL-inactivated RSV for three days. After three days IFN-γ (A) and IL-5 (B) were determined in the supernatants.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

4. 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 I IFN,

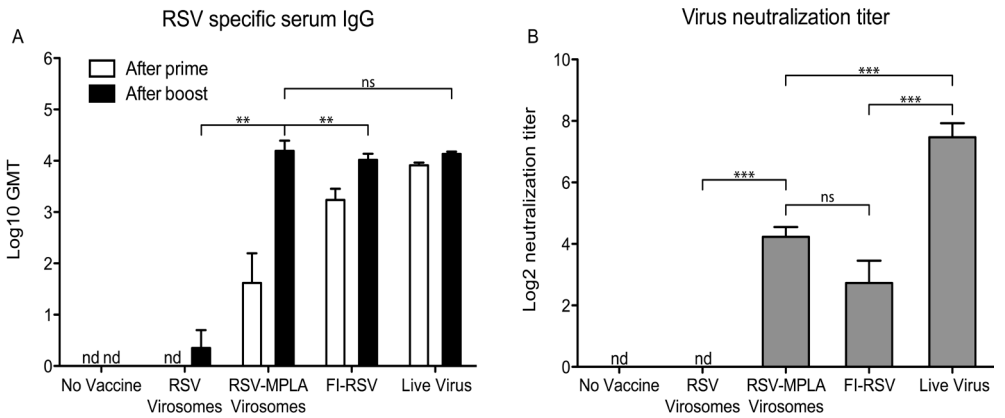

Figure 3. Immunogenicity in cotton rats. Cotton rats were immunized with the same preparations as given to mice (Figure 1) on day 0 and 21. On day 21 and 49, blood was taken en RSV specific IgG was determined in serum (A). RSV-virus neutralizing antibodies were determined in the day 49 serum (B). (Mann-Whitney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

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 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 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.

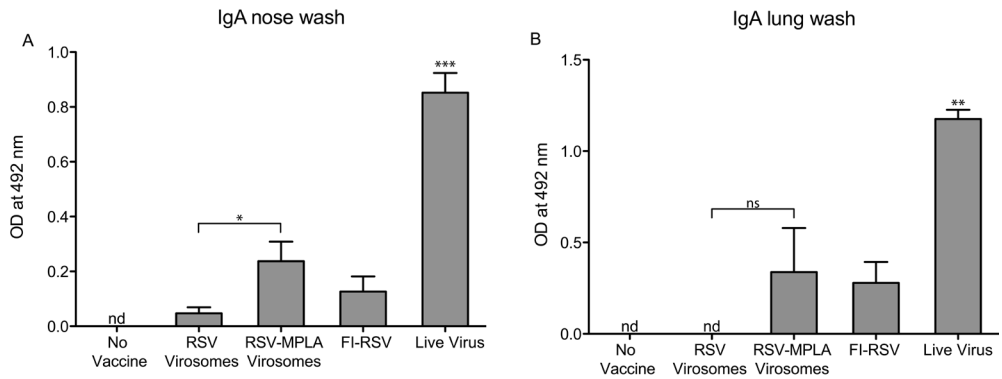

Figure 4. Mucosal immune response in cotton rats. Cotton rats were immunized as in Figure 3 and challenged with 1x 10^6 $TCID_{50}$ live RSV on day 49. Five days after challenge, the rats were s acrificed and lung and nose washes were taken. RSV specific IgA was determined in nose washes (A) and lung 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.

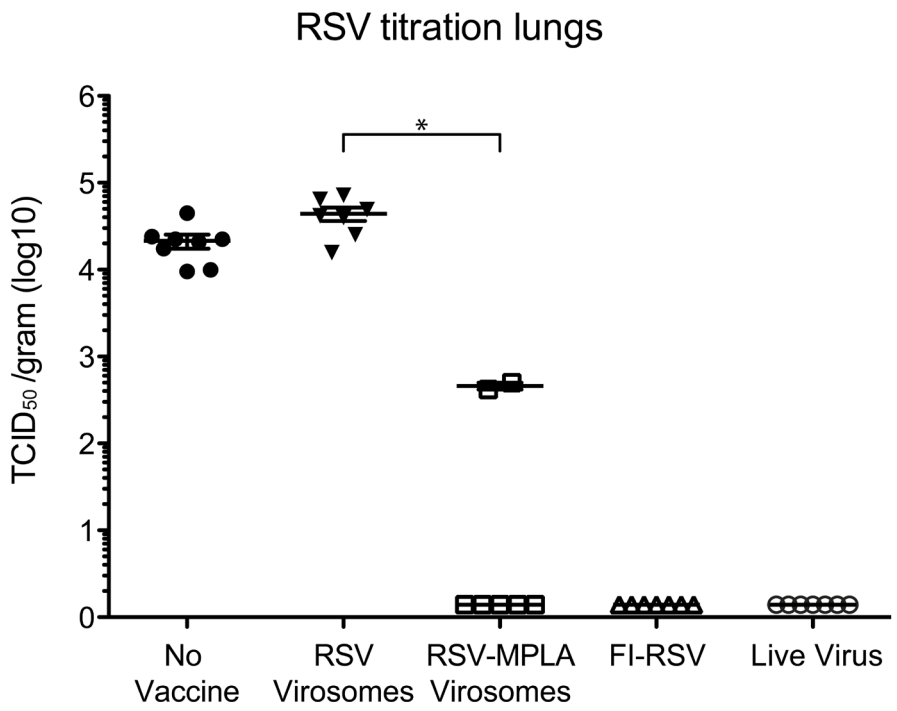

Figure 5. Protection in cotton rats. Cotton rats were immunized, challenged and sacrificed as in Figure 4. After termination, the lungs were removed and RSV titers in lungs were determined by $TCID_{50}$. (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001). Horizontal lines represent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

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

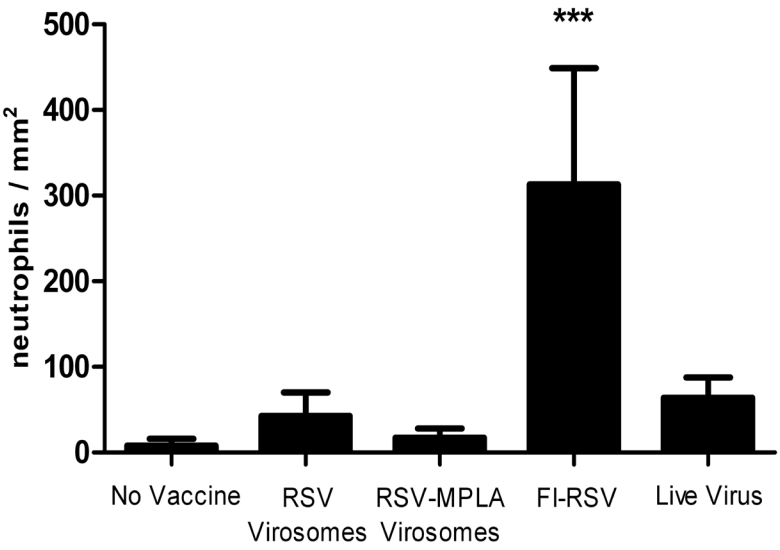

Figure 7. Neutrophil infiltration in lungs of immunized and RSV-challenged cotton rats. Cotton rats were immunized, challenged and sacrificed as in Figure 4. Lungs were removed, fixated and slices were stained with H & E. The numbers of infiltrating neutrophils were evaluated using light microscopy.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

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.

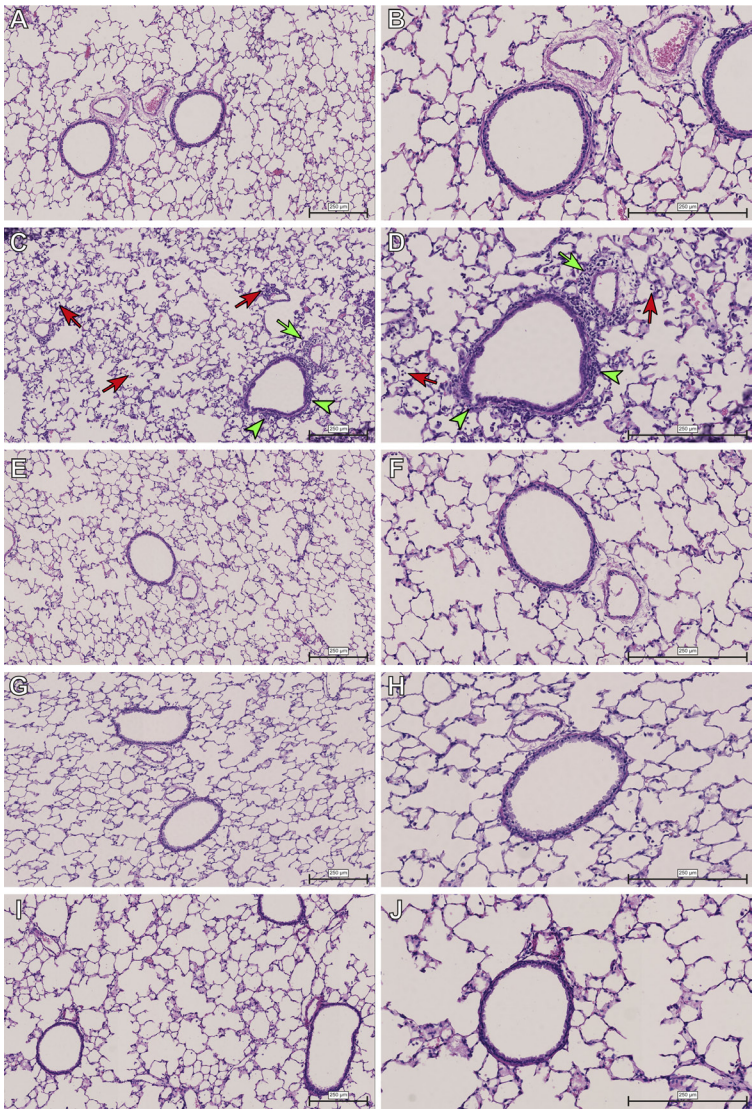

Figure 6. Lung pathology in cotton rats. Cotton rats were immunized, challenged and sacrificed as in Figure 4. After termination, one lung lobe was fixated with 4% formalin under 20 cm water pressure to retain the structure of the lung. After fixation, the lungs were embedded in paraffin and 4 µm slices 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.

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.

5. Acknowledgements

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6. 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, Blanco JCG. The 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 N. AS04, 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. American Journal of Respiratory Cell and Molecular Biology 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-κB 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, Small PA. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. J Virol 1991;65:2146–8.

Chapter 6

A virosomal Respiratory Syncytial Virus vaccine with incorporated Monophosphoryl Lipid A: Immunogenicity and protective efficacy in aged cotton rats after intramuscular or intranasal immunization

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Abstract

Each year, respiratory syncytial virus (RSV) infection is responsible for 180,000 hospitalizations and 14,000 deaths among the elderly in the USA alone, causing a disease burden similar to that of influenza in this population. Vaccination could significantly lower morbidity and mortality due to RSV among the elderly. Here, we investigated whether a virosomal RSV vaccine has the capacity to induce protective immune responses upon intramuscular (IM) injection in aged cotton rats and whether supplementation of RSV virosomes with monophosphoryl lipid A (MPLA) further stimulates antibody responses. Furthermore, we studied the induction and waning of serum antibody responses induced by a priming infection early in life and the capacity of virosomal RSV vaccine to boost these antibodies after ageing of the animals. Intramuscular (IM) injection of RSV virosomes induced protective RSV-specific serum IgG antibody responses in aged cotton rats. Immunization with RSV-MPLA virosomes further enhanced serum IgG levels compared to non-adjuvanted virosomes, without priming for enhanced lung pathology. RSV-specific serum antibodies, induced by infection early in life, waned significantly upon aging, but protective levels of specific antibody were still present at old age. RSV-specific serum antibody levels, however, increased after IM injection of RSV virosomes, irrespective of whether the virosomes were adjuvanted with MPLA or not. RSV-specific secretory IgA (S-IgA) in the respiratory tract of primed and aged cotton rats increased after intranasal (IN) immunization, but not after IM immunization, with virosomal RSV vaccine, and incorporation of MPLA in the virosomes boosted this response. Taken together these data show that RSV-MPLA virosomes are immunogenic in an aged immune system and are able to boost preexisting immunity against RSV.

1. Introduction

Respiratory syncytial virus (RSV) is the major cause of viral bronchiolitis in children and also a significant problem in the elderly. By the age of two, nearly all children have been infected with RSV at least once [1]. RSV infection does not lead to life-long protection and multiple reinfections occur throughout life. Previous studies pointed to RSV-specific serum IgG, but also mucosal secretory IgA (S-IgA), as protective effector mechanisms in RSV infection of adults [2]. In healthy adults with insufficient immunity, reinfections may lead to mild disease with common cold-like symptoms [3]. At older age, however, the immune system weakens and RSV infections can induce severe disease [4,5]. In the elderly, RSV is responsible for approximately 180,000 hospitalizations and 14,000 deaths each year in the USA alone [6]. Compared to influenza, RSV contributes to a similar extent to severe lower respiratory infections in the elderly [6]. Treatment of elderly suffering from RSV is mainly supportive and consist of administration of fluids and in some cases bronchodilatators [5]. A vaccine against RSV, however, could significantly reduce the burden of disease in the elderly.

We previously showed that reconstituted RSV membranes, adjuvanted with the Toll-Like Receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) represent a promising vaccine candidate. Studies in mice and cotton rats showed that RSV-MPLA virosomes induce high levels of serum RSV-specific, virus-neutralizing, antibodies and a balanced Th1/Th2 response [7]. Immunization of mice or cotton rats with RSV-MPLA virosomes does not prime for lung pathology (i.e. enhanced respiratory disease [8-10]) upon natural infection in contrast to immunization with formalin-inactivated RSV (FI-RSV). It is not known whether the RSV virosomal vaccine has the capacity to potentiate immune responses in an aged immune system and, if MPLA has the capacity to boost responses under these conditions.

A suitable animal model to study the efficacy of RSV vaccines is the cotton rat (*Sigmodon hispidus*). Cotton rats are more permissive than mice for RSV and show signs of enhanced disease lung pathology similar to that seen in humans [9,11,12]. However, RSV protection studies in aged cotton rats, as a model for the elderly, are scarce. One previous study showed that nine-month-old cotton rats have a more prolonged RSV infection compared to young cotton rats [13]. This coincides with a delay in cytokine expression patterns in the lungs of infected cotton rats [14]. These data indicate that aged cotton rats indeed show signs of compromised antiviral immunity and would be a suitable model to study immune responses induced by RSV vaccines, including (MPLA-adjuvanted) RSV virosomes.

Here, we investigated the efficacy and safety of an RSV virosomal vaccine in old cotton rats that were either naïve to RSV or primed for RSV-specific immunity by infection early in life, similar to the general situation in humans. We show that intramuscular (IM) injection of RSV virosomes induce protective antibody responses in aged cotton rats and that incorporation of MPLA in the virosomes significantly boosts these responses without priming for enhanced lung pathology. Immunity to RSV induced by infection early in

life, as demonstrated by RSV-specific serum antibodies, waned significantly but protective antibody levels were still detected at old age. Levels of RSV-specific serum antibodies could, however, be boosted by IM injection of RSV virosomes, whether or not adjuvanted with MPLA. Finally, levels of respiratory tract RSV-specific secretory IgA (S-IgA), a correlate of protection against RSV in the elderly, could be boosted by intranasal (IN) administration of RSV virosomes to primed and aged cotton rats and incorporation of MPLA in the virosomes boosted this response. These data together suggest that RSV-MPLA virosomes have the capacity to boost protective RSV-specific immune responses in an aged immune system. Therefore, RSV-MPLA virosomes represent a promising vaccine candidate RSV vaccine for the elderly.

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 Dutch Animal Protection Act (permit number DEC 5266E). Immunizations and challenges were conducted under isoflurane anesthesia, and every effort was made to minimize suffering of the animals.

2.2 Cells and virus

RSV strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in Vero cells (ATCC, CL-81, Wesel Germany): in SMF-4 Megavir (Thermo scientific, Etten-leur, The Netherlands), supplemented with L-glutamine, and penicilline/streptomycin (Invitrogen, Breda, Netherlands). After 5 days of infection the cell debris was cleared by low-speed centrifugation, the virus was concentrated and frozen in liquid nitrogen and stored at –80°C to be used for live virus immunizations, challenge and microneutralization assays.

2.3 Vaccine formulations

RSV virosomes were generated as described previously [15]. Briefly, the RSV membrane was dissolved in 100 mM 1,2 dicaproyl-sn-glycero-3-phosphocholine (DCPC) in 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, monophosphoryl lipid A from *Salmonella minnesota* Re 595 (Invivogen, Toulouse, France), was converted to 3-*o*-desacyl monophosphoryl lipid A by alkaline hydrolysis [16], dissolved in 100 mM DCPC in HNE, and 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 buffer pH 7.4. After dialysis, virosomes were kept at 4°C.

FI-RSV was produced as reported before [9]. FI-RSV was diluted with HNE to contain 5 µg of RSV protein in a 50 µL volume.

2.4 Animals and immunizations

Female outbred cotton rats (Hsd:Cotton Rat) of 18 to 21 days old were obtained from Harlan (Indianapolis, IN, USA). One group of animals was housed conventionally for 34 weeks before immunization. Another group of animals received an infection with 10^6 $TCID_{50}$ live RSV and was housed in individually ventilated cages (IVC). The infected animals showed no signs of illness after infection. Blood was sampled at 4 weeks post infection to determine the RSV-specific IgG response. In a number of animals, ageing was associated with development of disease symptoms including damaged eyes, abscess or tumor formation or group B Streptococcus infection of the uterus. Animals with more severe symptoms were terminated and excluded from the experiment. RSV infection at a young age did not correlate with the onset of the symptoms.

Cotton rats that were not infected at a young age were immunized at the age of 37 weeks (priming immunization) and 40 weeks (boosting immunization) with RSV virosomes or RSV-MPLA virosomes intramuscularly (IM; 50 µL). Virosome preparations contained 5 µg of viral protein. Control rats received 50 µL of HNE IM or 50 µL (diluted to contain 5 µg viral protein) of FI-RSV IM. At week 44, cotton rats were challenged with 10^6 $TCID_{50}$ RSV IN. Cotton rats that received a primary infection (referred to as RSV-primed cotton rats) were immunized once, at the age of 37 weeks with virosomes as described above. To assess the capacity of intranasally (IN) administered RSV virosomes to boost serum IgG responses and mucosal antibody responses in RSV-primed cotton rats, 100 µL (adjuvanted) virosomes were administered IN to anesthetized cotton rats. At 41 weeks, the cotton rats were challenged as described above.

At the time of immunization and challenge, blood was drawn by retro-orbital puncture. Five days after challenge, the cotton rats were sacrificed and blood was drawn by heart puncture. The cotton rats were resected and a small incision was made in the trachea. From this incision, nose washes and broncho-alveolar lavages were performed with 1 mL of PBS with protease inhibitors (Roche, Mannheim, Germany). Lungs were removed aseptically and one of the primary bronchi was ligated just below the tracheal bifurcation with suture wire. One half of each lung was kept on ice in HEp-2 medium containing 2% FBS, for virus titration. The other half of each lung was fixed in 4% formaldehyde in PBS at 20 cm of water pressure to preserve the structure of the lungs for lung histopathological analyses.

2.5 IgG antibody ELISA

RSV-specific antibody titers were determined as described before [15]. Briefly, 96-well plates were coated with β-propiolactone-inactivated RSV and blocked with 2.5% milk powder in coating buffer. Plates were then incubated for 90 min with two-fold serial dilutions of serum, BAL or nose washes, 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 are cross reactive with cotton rat IgG and IgA, respectively (Southern Biotech 1030-05, 1040-05) for 1 h and subsequently stained with *o*-phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA). After 30 min the staining was stopped by addition of 2M H_2SO_4 and absorption was measured at 492 nm.

2.6 Virus titration and microneutralization assay

Virus titers were determined by $TCID_{50}$ as described previously [7]. 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 [7]. The neutralization titer was calculated with the Reed & Muench method as the dilution that neutralizes infection in 50% of the wells.

2.7 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.

2.8 Statistical analyses

All statistical analyses were performed with Graphad Prism 5.00 for Mac OSX, (GraphPad Software, San Diego California 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.

3. Results

3.1 RSV-MPLA virosomes in RSV-naïve old cotton rats

To determine whether RSV virosomes or RSV-MPLA virosomes are immunogenic in cotton rats with an aged immune system, aged (i.e. 8-months-old) cotton rats were immunized twice intramuscular (IM) injections, three weeks apart, with RSV virosomes or RSV-MPLA virosomes. As controls, we included cotton rats that received no immunization or were immunized with FI-RSV by IM injection. We then analyzed levels of RSV-specific serum IgG, virus-neutralization capacities of sera obtained from the immunized cotton rats and protection against infection. Immunization with

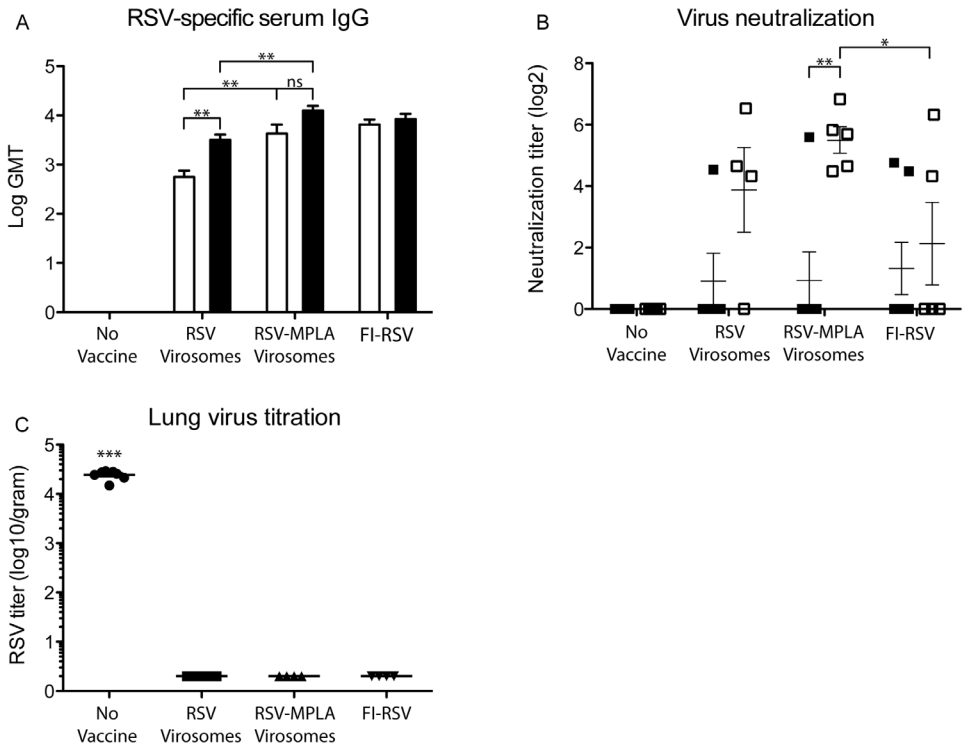

Figure 1. Immunogenicity and protection induced by vaccine in RSV-naive aged cotton rats. Cotton rats were immunized IM twice, once at 37 and once at 40 weeks old with RSV virosomes, RSV-MPLA virosomes, FI-RSV or live virus. Control mice were not vaccinated and received buffer. Fourteen days after the immunizations blood was drawn and RSV-specific IgG in serum was determined (A) (white bar:14 days after 1st immunization, black bar 14 days after second immunization). Serum virus-neutralization titers were determined after the first (black squares) and after the second immunization (white squares) by microneutralization assay (B). The immunized cotton rats were challenged on week 44 with 10^6 $TCID_{50}$ RSV and terminated 5 days later. After termination, RSV virus titers were determined by $TCID_{50}$ on lung homogenates (C). (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001)

RSV virosomes in aged cotton rats gave rise to RSV-specific serum IgG with a geometric mean titer (GMT) of 2.7 (Figure 1A). Incorporation of MPLA in the virosomes significantly enhanced the levels of RSV-specific serum IgG. These levels were comparable to those induced by FI-RSV. A second immunization further stimulated IgG responses in the group vaccinated with RSV virosomes, but the levels were lower than those induced by two immunizations with RSV-MPLA virosomes or FI-RSV (Figure 1A).

Antiviral antibody titers in the sera were also analyzed in an *in vitro* micro-neutralization assay (Figure 1B). A single immunization with RSV virosomes, RSV-MPLA virosomes or FI-RSV induced detectable neutralizing antibody titers only in a fraction of animals (Figure 1B). After a second immunization, the number of animals with detectable neutralizing antibody titers increased in the groups that received RSV virosomes, but not in the group that received FI-RSV. However, all animals in the RSV-MPLA-immunized group then developed neutralizing antibodies (Figure 1B).

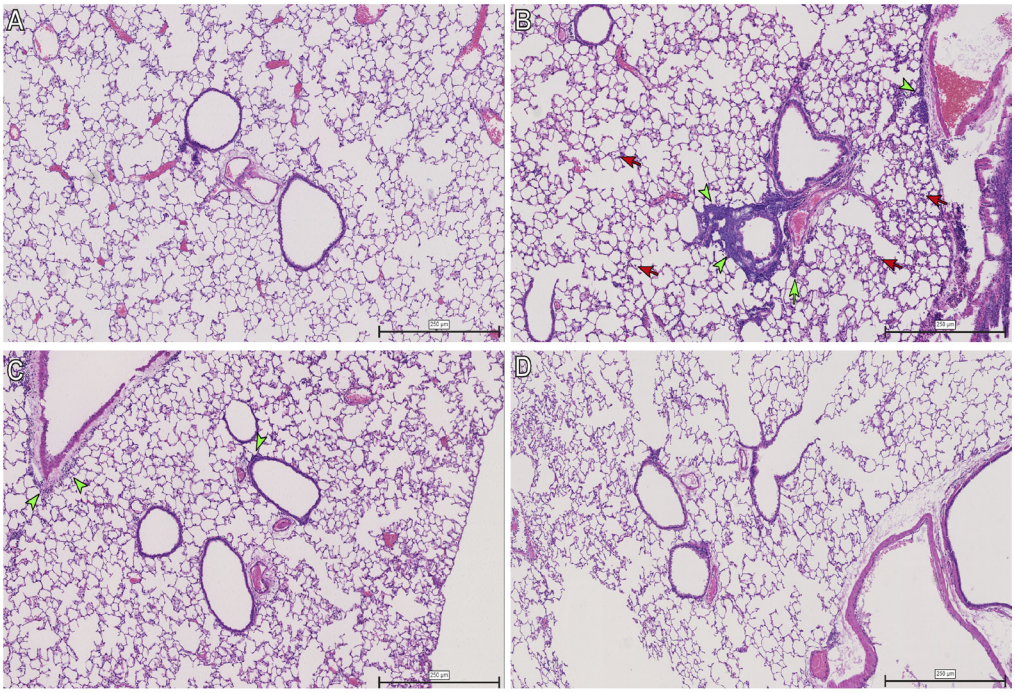

Figure 2. Lung pathology after immunization and challenge in aged cotton rats. The cotton rats were immunized challenged and terminated as in figure 1 with buffer (A), FI-RSV (B) RSV virosomes (C) and RSV-MPLA virosomes (D). The lungs were inflated and fixed with 4% formaldehyde and slices were stained with H&E. Lung pathology presenting as alveolitis and perivascular and peribronchial infiltrations was determined by light microscopy. Red arrows, alveolar infiltrates, green arrows, perivascular infiltrates, green arrowheads, peribronchial infiltrates.

The aged and immunized cotton rats were challenged with live RSV, 49 days after the first immunization, to analyze the protection afforded by vaccination. After five days, the animals were sacrificed and virus titers were measured in the lungs (Figure 1C). The lungs from non-immunized animals all had virus titers of, on average, 4.39 log $TCID_{50}$. Cotton rats that received FI-RSV, RSV virosomes or RSV-MPLA virosomes did not have detectable lung virus titers (Figure 1C).

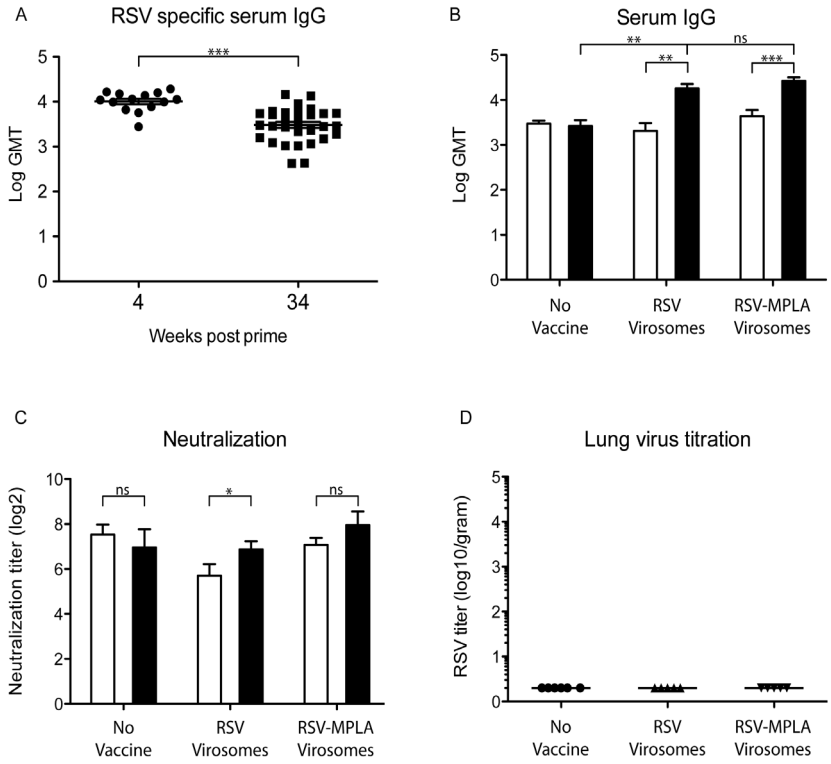

Figure 3. Immunogenicity and protection in RSV-primed and aged cotton rats immunized IM with RSV virosomes and RSV-MPLA virosomes. Cotton rats were infected with RSV at the age of 27 days. Four and 34 weeks after infection blood was drawn to determine RSV-specific serum IgG in 14 and all the animals respectively (A). At 37 weeks, the cotton rats were immunized with RSV virosomes or RSV-MPLA virosomes. Control cotton rat received buffer. Three weeks after immunization blood was drawn for RSV-specific serum IgG (B) and virus neutralization (C) determinations before (white bars) and after (black bars) immunization. The cotton rats were challenged with 10^6 $TCID_{50}$ RSV. Five days after challenge the cotton rats were terminated and RSV virus titers were determined in lung homogenates (D). (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001)

3.2 Enhanced respiratory disease

To determine the safety of RSV and RSV-MPLA virosomes with respect to immunopathology upon RSV infection following vaccination, we performed histopathological analyses on the lungs of immunized and challenged aged cotton rats. We compared the lungs from animals immunized with RSV virosomes and RSV-MPLA virosomes to the lungs of animals immunized with FI-RSV, which is known to prime for enhanced lung pathology [17]. The lungs from non-immunized aged cotton rats that were infected with RSV showed no signs of lung pathology: there were no alveolar infiltrates and no infiltration in the peribronchial and perivascular regions (Figure 2A). In contrast, aged cotton rats immunized with FI-RSV showed severe lung inflammation with peribronchial and perivascular infiltrates upon infection (Figure 2B). High numbers of neutrophils were also visible in the alveoli close to the bronchi. The lungs of the animals immunized with RSV virosomes showed some infiltrates in the peribronchial regions but no perivasculitis or alveolitis, as observed in animals immunized with FI-RSV. The lungs of aged cotton rats immunized with RSV-MPLA virosomes were similar to the lungs from non-immune animals and showed no signs of immunopathology upon infection (Figure 2D).

3.3 RSV virosomes in aged cotton rats infected with RSV early in life

The above data indicate that RSV-naïve aged cotton rats have an intact immune response, and are susceptible to priming for enhanced disease like young cotton rats. Practically all humans are exposed to RSV during childhood, and develop anti-RSV antibodies that remain present throughout life. To mimick this process, 27 day-old cotton rats were infected with RSV and, immunized with RSV virosomes 8 months later with or without MPLA as an adjuvant. Inoculation of live RSV to the young cotton rats induced an infection in all animals, as evidenced by the presence of RSV-specific serum IgG in all animals, 4 weeks after infection (Figure 3A). The levels of RSV-specific serum IgG were significantly decreased after 8 months, but still detectable in all animals (Figure 3A). At that time, the animals received a single IM injection with RSV virosomes or RSV-MPLA virosomes. A control group was not immunized. Three weeks after immunization, we observed significant increases in RSV-specific serum IgG levels with respect to pre-immunization levels (Figure 3B). RSV-specific serum IgG was not different for animals immunized with virosomes with or without adjuvant (Figure 3B). We also observed a significant increase in virus-neutralizing capacity of sera from the animals that were immunized with RSV virosomes (Figure 3C). The virus-neutralizing capacity of sera from RSV-MPLA-immunized animals also increased, but the enhancement did not reach a statistically significant difference, probably due to a higher base-line neutralizing titer in pre-immunization sera from animals that received RSV-MPLA virosomes (Figure 3C).

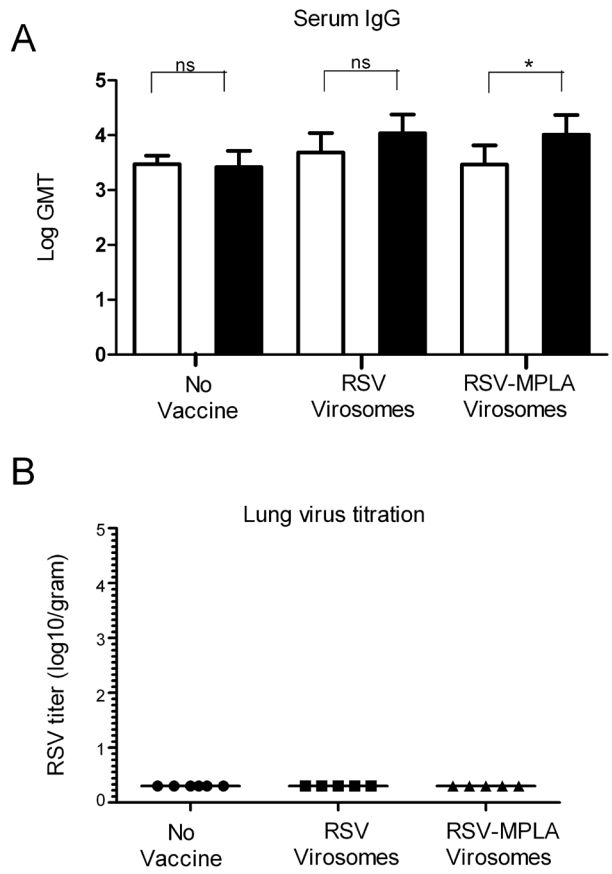

Figure 4. Immunogenicity and protection in RSV-primed and aged cotton rats immunized IN with RSV virosomes and RSV-MPLA virosomes. The cotton rats were infected and aged as in figure 3. At 37 weeks, the cotton rats were immunized IN with RSV virosomes or RSV-MPLA virosomes. Control cotton rat received buffer. Three weeks after immunization blood was drawn for RSV-specific serum IgG (A) determinations before (white bars) and after (black bars) immunization. The cotton rats were challenged with 10^6 $TCID_{50}$ RSV. Five days after challenge the cotton rats were terminated and RSV virus titers were determined in lung homogenates (B). (Mann-Whitney U test: ns not significant,*p<0.05)

Three weeks after immunization, the animals were challenged and five days later, RSV titers were determined in the lungs of the animals. RSV virus was not detected in the lungs from any group (Figure 3D). Therefore, RSV infection in young cotton rats induces immunity capable of protection against infection at 9-10 months.

3.4 Boosting of serum IgG and S-IgA antibodies levels by IN immunization in RSV-primed and aged cotton rats

IN administration of vaccines represents an attractive alternative to IM injection and may also induce mucosal S-IgA. The latter responses have also been linked with protection against RSV in the elderly [2]. We therefore determined if local IN administration of RSV virosomes or RSV-MPLA virosomes could boost serum IgG and mucosal S-IgA responses in RSV-primed and aged cotton rats. To this end, two groups of animals were immunized IN with RSV virosomes or RSV-MPLA virosomes. Three weeks after immunization, we observed significant increases in RSV-specific serum IgG levels in the group immunized IN with RSV-MPLA virosomes, but not in the group immunized IN with non-adjuvanted RSV virosomes (Figure 4A). At this time point, the animals were challenged and, five days later, RSV titers were determined in their lungs. Similar as in cotton rats immunized IM, RSV virus was not detected in the lungs of any of the animals (Figure 4B).

S-IgA responses were also analyzed in these groups and compared to responses seen in groups that received the vaccine IM. Lung and nasal washes were taken at time of sacrifice of the animals (i.e. 5 days post-challenge) to determine RSV-specific S-IgA. (Figure 5). IN immunization with RSV virosomes or RSV-MPLA virosomes resulted in significantly higher levels of S-IgA in lung and nasal washes as compared to those in washes from cotton rats that received the vaccine IM (Figure 5A and B). Incorporation of MPLA in IN-administered virosomes significantly boosted lung S-IgA levels, but not nasal S-IgA levels (Figure 5A and B). Thus, IN immunization with RSV-virosomes, adjuvanted with MPLA, is an effective approach to boost protective serum IgG and S-IgA antibody responses in RSV-primed and aged cotton rats.

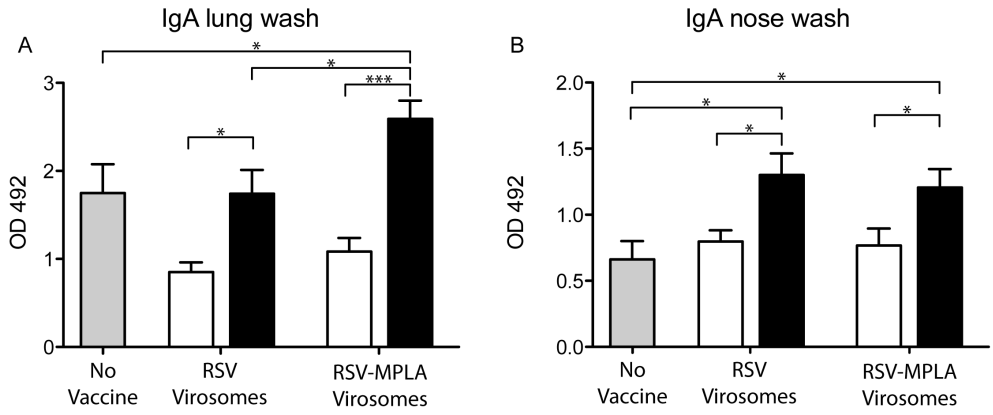

Figure 5. Mucosal IgA responses in RSV-primed and aged cotton rats immunized IM or IN with RSV virosomes and RSV-MPLA virosomes. The cotton rats were infected and aged as in figure 3. Subsequently the cotton rats were immunized with RSV virosomes and RSV-MPLA virosomes either intramuscularly (white bars) or intranasally (black bars). Control animals received buffer (grey bars). Three weeks after immunization the cotton rats were challenged and terminated. After termination, lung (A) and nose (B) washes were taken for RSV-specific IgA determination

4. Discussion

An efficacious RSV vaccine could significantly decrease morbidity and mortality in the elderly. Immunosenescence in the elderly causes them to gradually become more susceptible to infections and, at the same time, less responsive to vaccinations [18,19]. Here we show that RSV virosomes and RSV virosomes adjuvanted with MPLA are efficacious and safe in boosting RSV-specific immunity in aged cotton rats.

The adjuvant effect of the TLR4 ligand MPLA on vaccine-induced immune responses in RSV-naive aged cotton rats is similar to the effect seen in young cotton rats (manuscript submitted for publication). This means that TLR4-mediated signaling is still intact in aged cotton rats. The available knowledge of TLR expression and function in the aged immune system is mainly derived from studies in mice which have shown decreased expression [20] or decreased function of several TLR, including TLR4 [21,22]. One study in elderly people did show reduced a cytokine response to TLR1/2 ligands, but not to TLR4 ligands [23]. Therefore, differences may exist in the responses to different TLR ligands in aged immune systems from different species. An intact response to the TLR4 ligand MPLA in aged cotton rats, as shown in this study, but also in elderly people (17), suggests that MPLA is suitable as an adjuvant in RSV vaccines for the elderly.

Cotton rats that experienced an RSV infection early in life did not become susceptible for RSV at old age. We did observe a decline in serum IgG levels upon aging, however, this reduction was smaller than the decline reported in humans after infection [24] and the reduced levels if IgG were still able to afford protection. Co-evolution of RSV and the human immune system produced RSV strains capable of evading the human immune response to leave a previously infected person susceptible for subsequent infection. This is often referred to as 'incomplete' immunity induced by natural infection. Two possible mechanisms by which RSV inhibits the formation of effective immunity could involve inhibition of induction of type 1 IFN [25,26] or skewing the immune response to infection to a Th2-phenotype [27]. It is not clear if similar mechanisms are operational in RSV infection of cotton rats. Previous studies in cotton rats have shown, however, that RSV infection does not lead to a Th2-skewed response [11]. Also, cotton rats primed early in life were still protected against infection at old age (Figure 3), which argues against the presence of an efficient immune evading mechanism of RSV in cotton rats.

In primed and aged cotton rats, a similar increase in RSV-specific serum IgG was seen after IM immunization with RSV virosomes or RSV-MPLA virosomes. MPLA, although effective in aged cotton rats (Figure 1 and 5), does not seem to further boost serum IgG responses. An explanation for this may be the relatively high antibody levels in aged cotton rats induced by the infection early in life. Although these levels declined upon aging, they remained sufficiently high to provide protection. It is known that pre-existing antigen-specific antibodies negatively influence activation of B cells with the same antigen specificity, thereby avoiding unnecessary excess

antibody production. This mechanism is also operational after influenza vaccination. For example, vaccination with a similar strain as that used in the preceding years reduces further increases in antibody levels to that strain [28]. It is therefore likely that an enhancing effect of MPLA on serum antibody levels can only be observed when levels have sufficiently waned. RSV-MPLA virosomes administered IN to primed and aged cotton rats induce significant increases in serum IgG and respiratory tract S-IgA levels. A study in elderly people has shown that the levels of mucosal S-IgA correlated with protection against RSV infection [2]. Induction of S-IgA by vaccination is most efficiently induced by mucosal vaccination, e.g., IN immunization [29]. As the function of the nasal-associated lymphoid tissue remains relatively intact during aging, mucosal vaccination through the intranasal route seems attractive in, for example, approaches to induce (mucosal) immunity to RSV in the elderly [30]. In this respect, TLR ligands show promise as adjuvants in mucosal vaccines for the elderly [30]. In line with this, we found that the TLR4 ligand MPLA has the capacity to boost lung S-IgA responses upon IN immunization of aged cotton rats (Figure 5).

Taken together, our data indicate that RSV virosomes adjuvanted with MPLA have the capacity to induce and boost RSV-specific protective immune responses in an aged immune system. These data warrant further exploration of MPLA-adjuvanted RSV virosomes as a candidate vaccine for risk groups such as the elderly.

5. References

1. K.J. Henrickson, S. Hoover, K.S. Kehl, W. Hua, National disease burden of respiratory viruses detected in children by polymerase chain reaction, Pediatr Infect Dis J. 23 (2004) S11–8.
2. E.E. Walsh, A.R. Falsey, Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults, J Infect Dis. 190 (2004) 373–378.
3. C.B. Hall, C.E. Long, K.C. Schnabel, Respiratory syncytial virus infections in previously healthy working adults, Clin Infect Dis. 33 (2001) 792–796.
4. A.R. Falsey, Respiratory syncytial virus infection in adults, Semin Respir Crit Care Med. 28 (2007) 171–181.
5. E.E. Walsh, Respiratory syncytial virus infection in adults, Semin Respir Crit Care Med. 32 (2011) 423–432.
6. A.R. Falsey, P.A. Hennessey, M.A. Formica, C. Cox, E.E. Walsh, Respiratory syncytial virus infection in elderly and high-risk adults, N Engl J Med. 352 (2005) 1749–1759.
7. T. Kamphuis, T. Meijerhof, T. Stegmann, J. Lederhofer, J. Wilschut, A. de Haan, Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid a in mice, PLoS ONE. 7 (2012) e36812.
8. H.W. Kim, J.G. Canchola, C.D. Brandt, G. Pyles, R.M. Chanock, K. Jensen, et al., Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine, Am. J. Epidemiol. 89 (1969) 422–434.
9. G.A. Prince, S.J. Curtis, K.C. Yim, D.D. Porter, Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine, J Gen Virol. 82 (2001) 2881–2888.
10. M.E. Waris, C. Tsou, D.D. Erdman, S.R. Zaki, L.J. Anderson, 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. 70 (1996) 2852–2860.
11. M.S. Boukhvalova, G.A. Prince, J.C.G. Blanco, The cotton rat model of respiratory viral infections, Biologicals. 37 (2009) 152–159.
12. S. Niewiesk, G. Prince, Diversifying animal models: the use of hispid cotton rats (Sigmodon hispidus) in infectious diseases, Lab. Anim. 36 (2002) 357–372.
13. S.J. Curtis, M.G. Ottolini, D.D. Porter, G.A. Prince, Age-dependent replication of respiratory syncytial virus in the cotton rat, Exp. Biol. Med. (Maywood). 227 (2002) 799–802.
14. M.S. Boukhvalova, K.C. Yim, K.H. Kuhn, J.P. Hemming, G.A. Prince, D.D. Porter, et al., Age-related differences in pulmonary cytokine response to respiratory syncytial virus infection: modulation by anti-inflammatory and antiviral treatment, J Infect Dis. 195 (2007) 511–518.
15. T. Stegmann, T. Kamphuis, T. Meijerhof, E. Goud, A. de Haan, J. Wilschut, Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation, Vaccine. 28 (2010) 5543–5550.
16. N. Qureshi, K. Takayama, E. Ribi, Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium, J Biol Chem. 257 (1982) 11808–11815.
17. G.A. Prince, A.B. Jenson, V.G. Hemming, B.R. Murphy, E.E. Walsh, R.L. Horswood, et al., Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactiva ted virus, J Virol. 57 (1986) 721–728.
18. D. Aw, A.B. Silva, D.B. Palmer, Immunosenescence: emerging challenges for an ageing population, Immunology. 120 (2007) 435–446.
19. B. Grubeck-Loebenstein, S. Della Bella, A.M. Iorio, J.-P. Michel, G. Pawelec, R. Solana, Immunosenescence and vaccine failure in the elderly, Aging Clin Exp Res. 21 (2009) 201–209.
20. M. Renshaw, J. Rockwell, C. Engleman, A. Gewirtz, J. Katz, S. Sambhara, Cutting edge: impaired Toll-like receptor expression and function in aging, J Immunol. 169 (2002) 4697–4701.

21. E. Boehmer, J. Goral, D. Faunce, E. Kovacs, Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression, J Leukoc Biol. 75 (2004) 342–349.
22. E. Boehmer, M. Meehan, B. Cutro, E. Kovacs, Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway, Mech. Ageing Dev. 126 (2005) 1305–1313.
23. D. van Duin, S. Mohanty, V. Thomas, S. Ginter, R.R. Montgomery, E. Fikrig, et al., Age-associated defect in human TLR-1/2 function, J Immunol. 178 (2007) 970–975.
24. A.R. Falsey, H.K. Singh, E.E. Walsh, Serum antibody decay in adults following natural respiratory syncytial virus infection, J Med Virol. 78 (2006) 1493–1497.
25. M. Lo, R. Brazas, Respiratory Syncytial Virus Nonstructural Proteins NS1 and NS2 Mediate Inhibition of Stat2 Expression and Alpha/Beta Interferon Responsiveness, J Virol. (2005).
26. B. Bossert, S. Marozin, Nonstructural Proteins NS1 and NS2 of Bovine Respiratory Syncytial Virus Block Activation of Interferon Regulatory Factor 3, J Virol. (2003).
27. Y. Becker, Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review, Virus Genes. 33 (2006) 235–252.
28. W.E. Beyer, A.M. Palache, M.J. Sprenger, E. Hendriksen, J.J. Tukker, R. Darioli, et al., Effects of repeated annual influenza vaccination on vaccine sero-response in young and elderly adults, Vaccine. 14 (1996) 1331–1339.
29. I.M. Belyakov, J.D. Ahlers, What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? J Immunol. 183 (2009) 6883–6892.
30. K. Fujihashi, H. Kiyono, Mucosal immunosenescence: new developments and vaccines to control infectious diseases, Trends Immunol. 30 (2009) 334–343.

Chapter 7

General discussion

General discussion

Despite more than five decades of research, there is no licensed RSV vaccine available so far. This is partly due to the negative outcome of a clinical trial among infants in the 1960s using a formalin-inactivated, parentally administered, RSV vaccine, which did not confer protection but rather primed for enhanced respiratory disease (ERD) upon natural infection, to the extent that two of the vaccinated children even died after exposure to live RSV. Animal studies later showed that non-replicating vaccines, like (alum-adjuvanted) formalin-inactivated whole-virus RSV vaccine, wrongly instruct the immune system leading to, for example, non-neutralizing antibodies with low affinity and excessive Th2-skewed immune responses. With the recent discovery of Toll-like receptors (TLR) and other innate immune receptors as well as identification of their ligands, new opportunities for immunomodulation are now becoming increasingly available. For example, addition of TLR ligands to inactivated RSV vaccine antigens induce an altered immune activation upon immunization, leading to Th1-skewed responses and antibody responses with higher virus-neutralizing capacity, without priming for ERD. Recently, TLR ligands have also been recognized as potential mucosal immunoadjuvants. This would mean that TLR ligands could be employed in non-replicating RSV vaccines for mucosal delivery. Such approaches would represent attractive strategies for induction of RSV-specific immunity as mucosal (i.e. intranasal; IN) delivery is a highly accepted route of vaccine administration. Also, it could induce mucosal in addition to the systemic antibodies, which would further contribute to protection. In the studies on development of novel RSV vaccination strategies, described in this thesis, the IN route of immunization was explored using whole inactivated virus or reconstituted viral envelopes (virosomes) as non-replicating vaccine modalities. These vaccines were supplemented with different innate receptor ligands like TLR and/or NOD-like receptor (NLR) ligands. The capacity of different vaccine-incorporated innate receptor ligands to boost systemic and mucosal antibody responses, to induce safe Th1-type cellular responses and protection against infection, was studied in mice and (aged) cotton rats. Additionally, the safety of the vaccines was assessed by investigating the occurrence of ERD in immunized and subsequently challenged animals.

Mucosal vaccination strategies against RSV and immunoadjuvant properties of innate receptor ligands

IN administration of vaccines can induce, in addition to systemic immunity, local immune responses, i.e. secretory IgA (S-IgA), at mucosal sites, which could aid in the protection from infection with the pathogen involved [1-3]. In order to potentiate systemic and local antibody responses, non-replicating vaccines have to be supplemented with mucosal immunoadjuvants. As indicated above, TLR ligands form a new class of adjuvants that show

promise in this respect [4]. In **Chapters 2-5**, we show that IN immunization of mice and/or cotton rats with non-replicating RSV vaccine, supplemented with TLR ligands (e.g. MPLA, CpG or Pam_3CSK_4), significantly stimulates systemic and mucosal antibody responses. These immune responses were further augmented by adding a NOD2 ligand, i.e. L18-MDP (**Chapters 2-4**). TLR ligands exhibit their adjuvant function after binding to a wide range of cells within the mucosal tissue of the respiratory tract. These cells include not only cells of the immune system but also epithelial cells. Different TLR ligands, including ligands for TLR2 (Pam_3CSK_4), TLR4 (MPLA) or TLR9 (CpG DNA) ligands, can potentiate immune responses through induction of (pro-inflammatory) cytokines in macrophages and dendritic cells (DC), after binding to their specific TLRs, and engagement of the MyD88-mediated and/or TRIF-mediated signaling pathway [5]. Cytokines induced in these cell types, like IL-12 and type 1 IFNs, skew towards Th1-type responses with production of IFN-γ. These factors, contribute to antibody class switching to IgG2a [6]. Indeed, after mucosal co-administration of vaccines supplemented with TLR ligands, a switch towards the production of IgG2a was seen and splenocyte recall responses showed a clear skewing towards a Th1-type phenotype with IFN-γ production (**Chapters 2, 3 and 5**). Interestingly, different TLR ligands, including TLR9 ligand, induce B-lymphocyte-Activating Factor (BAFF) and A-Proliferation-Inducing-Ligand (APRIL) production in lung DCs and alveolar macrophages. These cytokines augment antibody responses upon interaction with B cells [7-9]. TLR ligands also directly interact with B cells. TLR ligands, such as TLR2, TLR4 and TLR9 ligands, can directly induce (T cell-independent) B cell activation and may help to induce mucosal antibody responses by enhancing class-switch recombination to IgA [10-14]. It is likely that direct activation of B cells by TLR ligands, mixed with inactivated RSV particles or incorporated in RSV virosomal membranes, contributed to the potentiation of S-IgA responses in the upper and lower respiratory tract (**Chapters 2-6**). Besides an activity on immune cells, TLR ligands may also act on other cell types, such as epithelial cells, endothelial cells and M-cells. Activation of epithelial cells by TLR ligands induces the release of certain pro-inflammatory cytokines and chemokines that can attract inflammatory cells. For example, activation of a human bronchial epithelial cell line (BEAS-2B) and primary human bronchial epithelial cells (PBEC) with TLR ligands significantly upregulated BAFF mRNA [15]. In another study, Sha *et al.* reported that stimulation of epithelial cells with TLR ligands, including TLR4 ligand, induced expression of macrophage inflammatory protein-3 (MIP-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which may help in DC maturation and migration [16]. A recent paper showed that Pam_3CSK_4, the TLR2 ligand used in the present study, not only up-regulates pro-inflammatory cytokine genes, but also genes involved in transendothelial migration of leukocytes at the site of vaccine administration [17]. Whether the latter mechanism would also be operational after mucosal immunization is not known. Finally, TLR ligands may enhance uptake of antigens by M cells, as has been described for TLR2 ligands [18,19] .

In the studies described in this thesis, a NOD2 ligand (L18-MDP) was used to further potentiate immune responses induced by the TLR ligands. This ligand has been reported to have mucosal immunoadjuvant activity [20], and can directly induce B cell activation in the absence of T cell help [21]. However, when incorporated in RSV virosomes, L18-MDP alone showed poor immunostimulation upon IN immunization of mice (**Chapter 3**). Interestingly, when added together with TLR ligands, it was effective in further boosting serum IgG and mucosal antibody responses in a synergistic fashion (**Chapter 2 and 3**). Possibly, these effects result from a boosting of the TLR signaling pathways by L18-MDP, rather than from induction of signaling events downstream of the NOD2 receptor, as described by Strober *et al*. [22].

In **Chapter 4**, we also observed an enhanced lung IgA response, in conjunction with systemic antibody responses, upon IN immunization of cotton rats with RSV virosomes carrying Pam_3CSK_4 and L18-MDP. Nasal IgA responses were poorly boosted, however. In contrast, IN immunization of mice with RSV virosomes, adjuvanted with Pam_3CSK_4/L18-MDP, did boost both lung IgA and nose IgA as well as serum IgG antibody responses (**Chapter 3**). A similar observation was made when IN virosomal RSV vaccine, adjuvanted with MPLA, was tested in cotton rats (**Chapter 5**). Currently, it is unclear why RSV virosomes adjuvanted with TLR ligands more efficiently induce nasal S-IgA in mice compared to cotton rats. Serum IgG and lung IgA responses in cotton rats were, however, effectively boosted by TLR ligands incorporated in RSV virosomes. Therefore, it is unlikely that the observed difference is due to a difference in a species-intrinsic capacity to respond to these ligands, as has been demonstrated, for example, for mouse vs. human cells in response to LpxL1, a TLR4 ligand [23]. An increase in the dose of adjuvants could perhaps boost nasal S-IgA to levels observed after RSV infection of cotton rats.

Role of T cells in vaccine-enhanced respiratory disease

The exact role of T cell responses associated with the FI-RSV vaccine-enhanced disease is not clear yet. Accumulated data from studies in mice suggest that Th2-associated immunity could be one of the possible factors responsible for disease enhancement [24-26]. Furthermore, previous studies have shown that FI-RSV-induced Th2-skewed cytokine responses (e.g. IL-5) in mice lead to infiltration of different cell types, including eosinophils, into the lungs upon infection [24]. The data described in **Chapter 2** show that mice vaccinated with FI-RSV not only had Th2-skewed splenocyte recall responses, but also had increased levels of IL-5 in their lungs at the time of RSV challenge infection. This may point to a role of Th2 cytokines in the observed lung pathology with eosinophil infiltration (**Chapter 2**), alveolitis, peribronchiolitis and perivasculitis (**Chapters 3-6**). IL-5, for example, can activate eosinophils while other Th2 cytokines (e.g. IL-4, IL-9 and IL-13) may upregulate VCAM-1, a mediator of lymphocyte and eosinophil migration [27].

Th2 responses could also play a role in activation/recruitment of IgE antibody-producing B cells, mast cells and eosinophils [28]. The data from **Chapter 3** show that mice vaccinated with (alum-adjuvanted) FI-RSV vaccine elicited a significantly enhanced induction of serum IgE antibody responses compared to these responses in mice immunized with other vaccine formulations. It is not unlikely that the Th2 cytokine IL-4 causes a switch towards IgE antibody production by differentiating B cells. On the other hand, the Th1 cytokine IFN-γ (a cytokine prominently produced upon immunization with TLR ligand-adjuvanted vaccine (**Chapters 2, 3 and 5**), can inhibit the switch to IgE and prevent the production of specific IgE. Th2 cells, through production of IL-4, IL-5, IL-9, and IL-13, are also important for mast cell recruitment, development, and function [29], which further contributes to immunopathology.

RSV vaccination and TLR activation under conditions of immunosenescence

RSV infection does not only affect infants, it is also a major cause of severe respiratory disease in the elderly [30]. It has been reported that RSV causes approximately 10,000 deaths annually in the United States in people over the age of 65 years [31]. Immunosenescence during aging not only makes elderly individuals more prone to infections but also suppresses the immune response to vaccines [32]. In order to investigate whether a candidate virosomal RSV vaccine, supplemented with a TLR4 ligand (MPLA), has the capacity to efficiently induce immune responses under conditions of potential immunosenescence, aged cotton rats were used in **Chapter 6.** RSV-MPLA virosomes effectively potentiated serum IgG responses upon IM administration, similar to responses observed in young cotton rats [33]. In aged cotton rats primed to RSV early in life, IN administration of RSV-MPLA virosomes could boost mucosal as well as systemic antibody responses (**Chapter 6**). Some studies, however, showed a decline in TLR expression and function with aging [34], which could imply that TLR ligands included in vaccines have a reduced adjuvant activity with increasing age. On the other hand, there is evidence showing that decreased cytokine production by human peripheral blood lymphocytes, activated with a TLR4 ligand (LPS), occurs only over the age of 85 years [35]. Therefore, the use of TLR ligands as adjuvants in vaccines for the elderly does appear to remain promising.

Future perspectives on RSV vaccination in specific target groups

Collectively, our data demonstrate that mucosal immunization with a virosomal RSV vaccine containing incorporated TLR and/or NOD2 ligands represents a promising approach to induce RSV-specific immunity. Upon IN administration, such adjuvanted candidate RSV vaccines induce protective mucosal as well as systemic antibody responses and Th1-skewed T cell responses in mice and (aged) cotton rats without priming for enhanced respiratory disease. The primary target groups for RSV vaccines are young infants and the elderly. In addition, older high-risk children would also benefit from RSV immunization. Furthermore, pregnant women may also represent a target group for vaccination with the aim to protect infants with maternal antibodies. It is likely that different vaccines will be required for these different target groups. Live-attenuated (LA) virus vaccines are likely to be best suited for use in RSV-naive infants, while non-replicating vaccines may be preferable for use in high-risk older children, pregnant women and the elderly [36]. The elderly represent the largest potential target group that may benefit from an adjuvanted virosomal RSV vaccine. Previous studies have shown that classical adjuvant systems, such as alum, have limited or no beneficial effects when co-administered with a non-replicating RSV vaccine [37]. TLR ligands are new-generation adjuvants that have shown to be highly efficacious in IM injected vaccines [38-40], and, based on the results presented in this thesis, show considerable promise for use in future non-replicating RSV vaccines for mucosal administration as well.

References

1. Holmgren J. Mucosal immunity and vaccination. FEMS Microbiol Immunol 1991;4(1):1-9.
2. Ogra PL, Faden H, Welliver RC. Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 2001;14(2):430-45.
3. Baca-Estrada ME, Foldvari M, Babiuk SL, Babiuk LA. Vaccine delivery: lipid-based delivery systems. J Biotechnol 2000;83(1-2):91-104.
4. Harandi AM, Medaglini D. Mucosal adjuvants. Curr HIV Res 2010;8(4):330-5.
5. Hennessy EJ, Parker AE, O'Neill LA. Targeting Toll-like receptors: emerging therapeutics? Nat Rev Drug Discov 2010;9(4):293-307.
6. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;236(4804):944-7.
7. Bessa J, Jegerlehner A, Hinton HJ, Pumpens P, Saudan P, Schneider P et al. Alveolar macrophages and lung dendritic cells sense RNA and drive mucosal IgA responses. J Immunol 2009;183(6):3788-99.
8. Katsenelson N, Kanswal S, Puig M, Mostowski H, Verthelyi D, Akkoyunlu M. Synthetic CpG oligodeoxynucleotides augment. Eur J Immunol 2007;37(7):1785-95.
9. Xu W, Santini PA, Matthews AJ, Chiu A, Plebani A, He B et al. Viral double-stranded RNA triggers Ig class switching by activating upper respiratory mucosa B cells through an innate TLR3 pathway involving BAFF. J Immunol 2008;181(1):276-87.
10. Liang Y, Hasturk H, Elliot J, Noronha A, Liu X, Wetzler LM et al. Toll-like receptor 2 induces mucosal homing receptor expression and IgA production by human B cells. Clin Immunol 2011;138(1):33-40.
11. Borsutzky S, Kretschmer K, Becker PD, Muhlradt PF, Kirschning CJ, Weiss S et al. The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. J Immunol 2005;174(10):6308-13.
12. Ozcan E, Garibyan L, Lee JJ, Bram RJ, Lam KP, Geha RS. Transmembrane activator, calcium modulator, and cyclophilin ligand interactor drives plasma cell differentiation in LPS-activated B cells. J Allergy Clin Immunol 2009;123(6):1277,86.e5.
13. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374(6522):546-9.
14. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci U S A 1996;93(7):2879-83.
15. Kato A, Truong-Tran AQ, Scott AL, Matsumoto K, Schleimer RP. Airway epithelial cells produce B cell-activating factor of TNF family by an IFN-beta-dependent mechanism. J Immunol 2006;177(10):7164-72.
16. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. Am J Respir Cell Mol Biol 2004;31(3):358-64.
17. Caproni E, Tritto E, Cortese M, Muzzi A, Mosca F, Monaci E et al. MF59 and Pam3CSK4 boost adaptive responses to influenza subunit vaccine through an IFN type I-independent mechanism of action. J Immunol 2012;188(7):3088-98.
18. Cashman SB, Morgan JG. Transcriptional analysis of Toll-like receptors expression in M cells. Mol Immunol 2009;47(2-3):365-72.
19. Chabot SM, Chernin TS, Shawi M, Wagner J, Farrant S, Burt DS et al. TLR2 activation by proteosomes promotes uptake of particulate vaccines at mucosal surfaces. Vaccine 2007;25(29):5348-58.
20. Fukushima A, Yoo YC, Yoshimatsu K, Matsuzawa K, Tamura M, Tono-oka S et al. Effect of MDP-Lys(L18) as a mucosal immunoadjuvant on protection of mucosal infections by Sendai virus and rotavirus. Vaccine 1996;14(0264-410; 0264-410; 6):485-91.
21. Petterson T, Jendholm J, Mansson A, Bjartell A, Riesbeck K, Cardell LO. Effects of NOD-like receptors in human B lymphocytes and crosstalk between NOD1/NOD2 and Toll-like receptors. J Leukoc Biol 2011;89(2):177-87.
22. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006;6(1):9-20.

23. Steeghs L, Keestra AM, van Mourik A, Uronen-Hansson H, van der Ley P, Callard R et al. Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of Neisseria meningitidis. Infect Immun 2008;76(8):3801-7.
24. 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(0022-538; 0022-538; 5):2852-60.
25. Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. J Immunol 1993;151(4):2032-40.
26. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. Immunol Rev 2011;239(1):149-66.
27. Steenwinckel V, Louahed J, Orabona C, Huaux F, Warnier G, McKenzie A et al. IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells. J Immunol 2007;178(5):3244-51.
28. Deo SS, Mistry KJ, Kakade AM, Niphadkar PV. Role played by Th2 type cytokines in IgE mediated allergy and asthma. Lung India 2010;27(2):66-71.
29. Ngoc PL, Gold DR, Tzianabos AO, Weiss ST, Celedon JC. Cytokines, allergy, and asthma. Curr Opin Allergy Clin Immunol 2005;5(2):161-6.
30. Walsh EE, Peterson DR, Falsey AR. Risk factors for severe respiratory syncytial virus infection in elderly persons. J Infect Dis 2004;189(2):233-8.
31. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ et al. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 2003;289(2):179-86.
32. Grubeck-Loebenstein B, Wick G. The aging of the immune system. Adv Immunol 2002;80:243-84.
33. Kamphuis T, Stegmann T, Meijerhof T, Wilschut J, De Haan A. A virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A provides protection against viral challenge without priming for enhanced disease in cotton rats. 2013. (Manuscript in revision).
34. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. J Immunol 2002;169(9):4697-701.
35. Van den Biggelaar AH, Huizinga TW, de Craen AJ, Gussekloo J, Heijmans BT, Frolich M et al. Impaired innate immunity predicts frailty in old age. The Leiden 85-plus study. Exp Gerontol 2004;39(9):1407-14.
36. Polack FP, Karron RA. The future of respiratory syncytial virus vaccine development. Pediatr Infect Dis J 2004;23(1 Suppl):S65-73.
37. Langley JM, Sales V, McGeer A, Guasparini R, Predy G, Meekison W et al. A dose-ranging study of a subunit Respiratory Syncytial Virus subtype A vaccine with and without aluminum phosphate adjuvantation in adults > or =65 years of age. Vaccine 2009;27(42):5913-9.
38. Descamps D, Hardt K, Spiessens B, Izurieta P, Verstraeten T, Breuer T et al. Safety of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine for cervical cancer prevention: a pooled analysis of 11 clinical trials. Hum Vaccin 2009;5(5):332-40.
39. Verstraeten T. Rebuttal Letter to the Letter to the Editor to "Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines" by T. Verstraeten et al. Vaccine 2009;27(19):2530.
40. Beran J. Safety and immunogenicity of a new hepatitis B vaccine for the protection of patients with renal insufficiency including pre-haemodialysis and haemodialysis patients. Expert Opin Biol Ther 2008;8(2):235-47.

Chapter 8

Appendices

Summary

Nederlandse samenvatting

Acknowledgements

List of publications

Summary

Respiratory syncytial virus (RSV) infection is a major cause of severe respiratory disease in infants and children worldwide and also forms a serious threat for the elderly. Vaccination could significantly relieve the burden of RSV disease. However, unfortunately, there is no licensed RSV vaccine available so far. RSV vaccine development has been hampered by the dramatic outcome of a clinical trial among infants in the 1960s with a formalin-inactivated, parentally administered, RSV vaccine, which did not confer protection. Rather, the vaccine primed for enhanced respiratory disease (ERD) upon natural infection, to the extent that two of the vaccinated children died after exposure to live RSV. The development of an RSV vaccine for mucosal administration, for example intranasal (IN) administration, is of great interest and would represent an attractive alternative to intramuscular (IM) injection. Intranasal delivery is a non-invasive and readily accepted route of administration and it can, in addition to systemic antibodies, induce local secretory-IgA (S-IgA) that may further contribute to protection. Also, mucosal immunization does not readily prime for ERD. However, since mucosally administered antigens are generally not very immunogenic, adjuvants are likely to be needed in intranasal vaccine. Recently, innate receptor ligands, including Toll-like receptor (TLR) ligands and NOD-like receptor (NLR) ligands, have been recognized as potential mucosal immunoadjuvants. In this thesis, an approach towards mucosal vaccination against RSV was explored which uses intranasal administration of RSV vaccine (inactivated RSV or RSV virosomes) supplemented with TLR and/or NLR ligands.

In **Chapter 2**, we demonstrate that mucosal immunization of mice with RSV whole virions, inactivated with β-propiolactone (BPL-RSV) and supplemented with innate receptor ligands (TLR9 ligand CpG ODN and NOD2 ligand L18-MDP), through the upper or total respiratory tract is an effective and safe approach to induce RSV-specific immunity. First, the data show that BPL-RSV, supplemented with CpG ODN and L18-MDP, potentiates activation of antigen-presenting cells (APC) *in vitro*, as demonstrated by NF-κB induction in a model APC cell line. *In vivo*, BPL-RSV, supplemented with CpG ODN/L18-MDP, induces local IgA responses and augments Th1-signature IgG2a subtype responses after immunization of the total respiratory tract (TRT) or the upper respiratory tract (intranasal, IN), albeit less efficiently via the latter route. Addition of TLR9/NOD2 ligands to the inactivated RSV also promoted affinity maturation of RSV-specific IgG antibodies and shifted T cell responses from mainly IL-5-secreting cells to predominantly IFN-γ-producing cells, indicating a Th1-skewed response. This effect was seen for both IN and TRT immunization. Finally, BPL-RSV, supplemented with TLR9/NOD2 ligands, significantly improved the protection of mice against a challenge with infectious virus, without stimulating ERD, as evidenced by the lack of eotaxin mRNA expression and eosinophil infiltration in the lungs of the animals.

In **Chapter 3**, the capacity of a virosomal RSV vaccine with incorporated lipophilic TLR and/or NOD2 ligand has been explored *in vitro* and *in vivo* in mice. Incorporation of Pam_3CSK_4 (a TLR2 ligand) and/or L18-MDP (a NOD2 ligand; see above) potentiated the capacity of the virosomes to activate (antigen-presenting) cells *in vitro*, as demonstrated by NF-κB induction. *In vivo*, incorporation of Pam_3CSK_4 in virosomes boosted serum IgG antibody responses and mucosal antibody responses after IN immunization of mice. While L18-MDP incorporated in RSV virosomes was not effective, incorporation of L18-MDP in Pam_3CSK_4-containing virosomes further boosted mucosal antibody responses. Finally, IN immunization with adjuvanted virosomes protected mice against infection with RSV, without priming for enhanced disease.

In **Chapter 4,** the efficacy of an IN virosomal RSV vaccine, with incorporated Pam_3CSK_4 or Pam_3CSK_4 together with L18-MDP, was investigated in cotton rats. The cotton rat represents an animal model that is more permissive for RSV infection in comparison with mice. The responses induced by the virosomal vaccine were compared with responses induced by IM immunization with a mock-up of the 1960s FI-RSV vaccine (alum-adjuvanted FI-RSV) and IN immunization with live virus. Incorporation of Pam_3CSK_4 in RSV virosomes potentiated systemic IgG and lung S-IgA antibody responses capable of inhibiting viral replication in cotton rat lungs upon challenge. Inclusion of L18-MDP in virosomes carrying Pam_3CSK_4, further potentiated these responses. Immunization with live virus, but not IN immunization with adjuvanted virosomes or intramuscular (IM) immunization with FI-RSV, induced nasal S-IgA. All immunized groups showed reduced viral lung titers upon challenge. Immunization with FI-RSV, but not IN immunization with adjuvanted virosomes or live virus, induced immunopathology (ERD) after challenge with live virus.

In **Chapter 5**, the efficacy and safety of RSV virosomes with incorporated monophosphoryl lipid A (MPLA, a TLR4 ligand) was studied after IN immunization of mice and cotton rats. MPLA is the only TLR ligand that is registered for use in human vaccines and holds promise for use in an IN virosomal RSV vaccine. In mice, we found that incorporation of MPLA in IN-administered RSV virosomes stimulated both systemic IgG and local S-IgA antibody levels as well as Th1-type skewing, and resulted in significantly reduced lung viral titers upon live virus challenge. Again, responses induced by the IN virosomal vaccine were compared with responses induced by IM immunization with FI-RSV vaccine and IN immunization with live virus. Antibody responses and Th1/Th2-cytokine responses induced by RSV-MPLA virosomes were comparable to those induced by live RSV infection. FI-RSV induced serum IgG, which inhibited viral shedding upon challenge, but also induced unfavourable 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 S-IgA responses were only moderately enhanced. Importantly, IN immunization with RSV-MPLA virosomes, like live virus infection, did not prime for ERD upon live virus challenge of immunized animals. In contrast, IM-immunization with FI-RSV induced severe lung immunopathology.

In **Chapter 6**, we tested the immunogenicity and safety of an MPLA-adjuvanted virosomal RSV vaccine in aged cotton rats as a model for the elderly human population in which RSV represents a serious health threat. Here, responses were studied upon IM and IN administration of vaccine to animals that were either RSV-naive or RSV-primed through RSV infection earlier in life. IM injection of RSV virosomes induced protective RSV-specific serum IgG antibody responses in aged cotton rats. Immunization with RSV-MPLA virosomes further enhanced serum IgG levels compared to non-adjuvanted virosomes, without priming for enhanced lung pathology. RSV-specific serum antibodies, induced by infection early in life, waned significantly upon aging. However, protective levels of antibody were still present at old age. RSV-specific serum antibody levels did increase after IM injection of RSV virosomes, irrespective of whether the virosomes were adjuvanted with MPLA or not. After IN administration, virosomes adjuvanted with MPLA, but not non-adjuvanted RSV virosomes, significantly enhanced serum IgG levels in RSV-primed and aged cotton rats. RSV-specific S-IgA in the respiratory tract of RSV-primed and aged cotton rats increased after IN immunization, but not after IM immunization, with virosomal RSV vaccine. Incorporation of MPLA in the virosomes boosted this response.

Finally, **Chapter 7** presents a discussion of the research described in this thesis and describes future perspectives for RSV vaccination in specific target groups.

Nederlandse samenvatting

Respiratoir syncytieel virus (RSV) is een belangrijke veroorzaker van ernstige luchtweginfecties bij pasgeborenen en jonge kinderen en vormt ook een bedreiging voor ouderen. Vaccinatie tegen RSV zou infectie en complicaties veroorzaakt door het virus kunnen verminderen, maar tot nu toe is er geen RSV vaccin beschikbaar. De ontwikkeling van een RSV vaccin is mede vertraagd door de desastreuze uitkomst van een klinische vaccinatiestudie in de zestiger jaren van de vorige eeuw. In deze studie werd een formaline-geïnactiveerd vaccin gebruikt. Dit vaccin bleek geen bescherming teweeg te brengen, maar induceerde juist immunopathologie (”enhanced disease”) na natuurlijke infectie van de gevaccineerde kinderen met RSV, als gevolg waarvan twee jonge kinderen kwamen te overlijden. De ontwikkeling van een nieuw veilig RSV vaccin blijft echter onverminderd is belangrijk.

Mucosale toediening zou een goed uitgangspunt kunnen vormen voor een effectief RSV vaccin. Mucosale toediening, zoals bijvoorbeeld intranasale (IN) toediening met een neusspray, is een geaccepteerde, niet-invasieve, toedieningsroute. Via deze route kunnen, naast RSV-specifieke serum IgG antistoffen, ook lokale IgA antistoffen opgewekt worden, die zouden kunnen bijdragen aan bescherming. Mucosale toediening van vaccin lijkt ook minder snel “enhanced disease” te veroorzaken. Om via de mucosale route effectieve immuunresponsen te induceren zijn echter adjuvantia nodig. Liganden voor “Toll-like” receptoren (TLRs) of “NOD-like” receptoren (NLRs) vormen een veelbelovende categorie nieuwe adjuvantia, die in een geïnactiveerd RSV vaccin gebruikt zouden kunnen worden. In dit proefschrift is onderzocht of intranasale toediening van een geïnactiveerd RSV vaccin, (zoals geïnactiveerde maar verder intacte RSV-virusdeeltjes of virosomen afgeleid van RSV), gesupplementeerd met TLR en/of NOD liganden, een geschikt methode is om op een veilige manier een beschermende immuunrespons tegen RSV op te wekken bij proefdieren zoals muizen en katoenratten (“cotton rats”).

In **Hoofdstuk 2** wordt beschreven zien dat intranasale toediening aan muizen van RSV, geïnactiveerd met β-propiolactone (BPL-RSV) en gesupplementeerd met een TLR9 ligand (CpG) en een NOD2 ligand (L18-MDP), een effectieve en veilige manier is om RSV-specifieke immuniteit te induceren. De resultaten lieten allereerst zien dat BPL-RSV, gesupplementeerd met CpG en L18-MDP, de activatie van antigeen-presenterende cellen *in vitro* stimuleert. Na IN toediening aan muizen induceert deze formulering ook een lokale IgA respons in de luchtwegen; ook werd een verhoogde productie van Th1-type IgG2a subklasse antistoffen waargenomen. TLR9 en NOD2 liganden bleken daarnaast de affiniteit van RSV-specifieke IgG antistoffen te bevorderen. Tenslotte werd een verschuiving in het type T cel respons waargenomen van een IL-5-producerende T cel naar een IFN-γ-producerende T cel. Dit betekent dat er voornamelijk een Th1-type respons wordt opgewekt. Dit effect was te zien na toediening van de formulering in de neus of de gehele luchtwegen. Toevoeging van de TLR9/NOD2 liganden aan

BPL-RSV bleek de vaccin-geïnduceerde bescherming tegen een "challenge" met infectieus virus significant te verbeteren. Hierbij werd geen expressie van mRNA voor eotaxine waargenomen en er werd ook geen infiltratie in de long van eosinofiele cellen gedetecteerd: er wordt dus geen "enhanced disease" geïnduceerd.

In **Hoofdstuk 3** werd een virosomaal RSV vaccin onderzocht. Virosomen zijn gereconstitueerde virusmembranen. Omdat deze deeltjes geen genoom meer bevatten kunnen ze niet repliceren. In deze studie werden lipofiele TLR en/of NOD2 liganden in de virosomale membraan ingebouwd en werd de immuunpotentiërende capaciteit van deze deeltjes *in vitro* en *in vivo* (bij muizen) onderzocht. Inbouw van een TLR2 ligand (Pam_3CSK_4) en/of een NOD2 ligand (L18-MDP) bleek de capaciteit van de virosomen om, *in vitro*, (antigeen-presenterende) cellen te activeren te versterken. Dit werd vastgesteld door de inductie van NF-κB te meten. Inbouw van Pam_3CSK_4 in de virosomen stimuleert ook de IgG antistofrespons en de mucosale IgA antistofrespons na IN toediening van het vaccin aan muizen. Inbouw van L18-MDP alleen bleek niet effectief te zijn. Echter, bij inbouw van L18-MDP in virosomen waarin tevens Pam_3CSK_4 aanwezig was werd wel een verhoging van de mucosale antistofrespons waargenomen. Tenslotte bleek IN immunizatie van muizen met RSV virosomen met ingebouwde lipofiele TLR/NOD2 liganden, volledige bescherming tegen een "challenge" met actief RSV virus te induceren, waarbij er geen immunopathologische afwijkingen (duidend op "enhanced disease") werden geconstateerd.

In **Hoofdstuk 4** werd het IN virosomale RSV vaccin, met en zonder ingebouwd Pam_3CSK_4/L18-MDP, onderzocht bij katoenratten ("cotton rats"). Cotton rats zijn proefdieren die, in vergelijking met muizen, verhoogd permissief zijn voor RSV-infectie. De immuunresponsen geïnduceerd door IN-toegediend virosomaal RSV vaccin werden vergeleken met de responsen geïnduceerd door IM-toegediend FI-RSV vaccin en IN immunisatie met actief virus. Inbouw van Pam_3CSK_4 in de RSV virosomen bleek de serum IgG en de lokale S-IgA respons in de longen te versterken. Inbouw van L18-MDP in virosomen waarin ook Pam_3CSK_4 was ingebouwd, versterkte de antistofresponsen verder. IN immunisatie met actief virus, maar niet IN immunisatie met (geadjuvanteerde) virosomen of IM immunisate met FI-RSV, bleek een S-IgA respons in de neusholte van cotton rats teweeg te brengen. Bij alle geïmmuniseerde groupen werd een verlaagde virustiter in de longen gemeten na "challenge" met actief levend virus. Immunisatie met FI-RSV, maar niet IN immunisatie met (geadjuvanteerde) virosomen of actief virus, bleek immunopathologie, karakteristiek voor "enhanced disease", te induceren na "challenge" met levend virus.

In **Hoofdstuk 5** werd een virosomaal RSV vaccin onderzocht waarin het lipofiele monophosphoryl lipid A (MPLA, een TLR4 ligand) was geïncorporeerd. MPLA is het enige geregistreerde TLR ligand dat momenteel gebruikt wordt in humane vaccins. Daarom was het van belang om te onderzoeken of MPLA ook gebruikt zou kunnen worden in een IN virosomaal RSV vaccin. Na IN toediening van het vaccin aan muizen vonden we dat virosoom-geïncorporeerd MPLA zowel de serum IgG respons als de lokale S-IgA respons in de luchtwegen versterkt. MPLA bleek ook een verschuiving

naar een Th1-type respons te induceren. Daarnaast werd bescherming tegen een "challenge" met actief virus waargenomen. De immuunresponsen werden ook in deze studie vergeleken met de responsen geïnduceerd door IM immunisatie met FI-RSV vaccin en IN immunisatie met actief virus. De antistofresponsen en Th1/Th2-cytokine responsen geïnduceerd door RSV-MPLA virosomen bleken vergelijkbaar te zijn met de responsen geïnduceerd door IN immunisatie met actief virus. IM immunisatie met FI-RSV bleek antistofresponsen te induceren die de virusreplicatie in de longen konden terugdringen, maar het FI-RSV vaccin bleek ook een ongwenste Th2-type respons op gang te brengen. Ook in cotton rats bleken RSV-MPLA virosomen een versterkte serum antistofrespons te induceren en virustiters in de longen na "challenge"met actief virus te verlagen. Inbouw van MPLA in de virosomen gaf echter maar een geringe versterking van de mucosale S-IgA respons te zien. IN immunisatie met RSV-MPLA virosomen veroorzaakt geen immunopathologie na "challenge" met actief virus en lijkt dus veilig voor wat betreft de mogelijke inductie van "enhanced disease".

In **Hoofdstuk 6** werd de immunogeniciteit en veiligheid van het virosomale RSV vaccin, met en zonder ingebouwd MPLA, getest in oude cotton rats. Omdat ouderen een belangrijke doelgroep voor een vaccinatie tegen RSV vormen, was het van belang om te onderzoeken wat het effect van het vaccin op een verouderend immuunsysteem zou zijn. Er werden immuunresponsen gemeten na zowel IM als IN toediening van het vaccin aan oude cotton rats. Hierbij werden twee verschillende groepen getest: RSV-naïeve cotton rats and cotton rats die op jonge leeftijd met RSV waren geïnfecteerd. IM toediening van RSV virosomen bleek een beschermende serum IgG antistofrespons in de RSV-naïeve cotton rats te induceren. Inbouw van MPLA in de virosomen versterkte deze beschermende respons en de geïnduceerde responsen gaven ook geen aanleiding tot "enhanced disease" na "challenge" van de dieren met actief virus. In de cotton rats die op jonge leeftijd geïnfecteerd waren met RSV, bleken de concentratie van RSV-specifieke serum IgG antistoffen significant verlaagd te zijn na de veroudering van de dieren. De spiegels van deze antistoffen bleek verhoogd te kunnen worden door vaccinatie van de dieren via IM toediening van RSV-virosomen, onafhankelijk van de inbouw van MPLA in de virosomen. Na IN toediening van virosomen met daarin ingebouwd MPLA, maar niet na IN toediening van virosomen zonder MPLA, konden de RSV-specifieke serum IgG spiegels ook worden verhoogd in deze groep. De concentratie RSV-specifiek S-IgA in de luchtwegen werd hierbij ook verhoogd, wat niet werd waargenomen na IM toediening van het vaccin. Inbouw van MPLA in de virosomen bleek deze S-IgA respons te versterken.

In **Hoofdstuk 7** worden de in dit proefschrift behaalde resultaten bediscussieerd en worden perspectieven voor toekomstige RSV vaccinatie in verschillende doelgroepen besproken.

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List of publications

1. **Shafique M**, Jan Wilschut and Aalzen de Haan. Induction of mucosal and systemic immunity against Respiratory Syncytial Virus by inactivated virus supplemented with TLR9 and NOD2 ligands

   Vaccine (2012) 30(3):597-606

2. **Shafique M**, Tjarko Meijerhof, Jan Wilschut and Aalzen de Haan. Evaluation of an intranasal virosomal vaccine against Respiratory Syncytial Virus in mice: Effect of TLR2 and NOD2 ligands on induction of systemic and mucosal immune responses

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3. Kamphuis T, **Shafique M**, Tjarko Meijerhof, Toon Stegmann, Jan Wilschut, Aalzen de Haan. Efficacy and safety of an intranasal virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A in mice and cotton rats

   Accepted in Vaccine

4. **Shafique M**, Tjarko Meijerhof, Jan Wilschut and Aalzen de Haan. An intranasal virosomal vaccine against Respiratory Syncytial Virus, supplemented with TLR2 and NOD2 ligands, induces RSV-specific systemic and mucosal immunity in cotton rats

   Manuscript in preparation

5. Kamphuis T, **Shafique M**, Tjarko Meijerhof, Toon Stegmann, Jan Wilschut, Aalzen de Haan. A virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A: Immunogenicity and protective efficacy in aged cotton rats after intramuscular or intranasal immunization

   Manuscript in preparation

6. **Shafique, M**, Ahmad N, Awan FR, Mustafa T, Ullah M, and Qureshi JA. Investigating the concurrent presence of HCV in serum, oral fluid and urine samples from chronic HCV patients in Faisalabad, Pakistan

   Arch Virol. (2009). 154(9):1523-1527

7. Ahmad, N., Asghar, M. **Shafique, M**. and Qureshi, J.A. An Evidence of High Incidence of Hepatitis C Virus in Faisalabad, Pakistan

   Saudi Med J. (2007). 28(3):390-395