Antiviral Research 162 (2019) 54–60

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Maternal vaccination with a novel chimeric glycoprotein formulated with a polymer-based adjuvant provides protection from human parainfluenza virus type 3 in newborn lambs

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ARTICLE INFO

Keywords:
Parainfluenza virus-3
Respiratory syncytial virus
Animal model
Maternal vaccination
Maternal antibodies
Newborn lambs

ABSTRACT

Human parainfluenza virus 3 (PIV3) and respiratory syncytial virus (RSV) are major causative agents of serious respiratory tract illness in newborns and infants. Maternal vaccination could be a promising approach to provide immediate protection against severe PIV3 and RSV infection in young infants. Previously, we demonstrated that maternal immunization with a subunit vaccine consisting of the RSV fusion (F) protein formulated with TriAdj, an adjuvant consisting of poly(I:C), immune defense regulatory peptide and polyphosphazene, protects newborn lambs from RSV. In the present study we evaluated the protective efficacy of a novel bivalent RSV-PIV3 vaccine candidate, F_{RipSc}HN/TriAdj, as a maternal vaccine against PIV3 infection in a neonatal lamb model. This vaccine consists of the pre-fusion form of the RSV F protein linked to the haemagglutinin-neuraminidase (HN) of PIV3, formulated with TriAdj. First, we successfully established PIV3 infection in neonatal lambs. Lambs infected with human PIV3 showed gross pathology, bronchointerstitial pneumonia and viral replication in the lungs. Subsequently, ewes were immunized with F_{RipSc}HN/TriAdj. RSV F_{RipSc}- and PIV3 HN-specific antibodies with virus-neutralizing activity were detected in both the serum and the colostrum of the vaccinated ewes. The newborn lambs had RSV- and PIV3- neutralizing antibodies in their serum, which demonstrates that maternal antibodies were transferred to the neonates. At three days of age, the newborn lambs received an intrapulmonary challenge with PIV3. The lung pathology and virus production were significantly reduced in lambs that had received PIV3-specific maternal antibodies compared to lambs born to non-vaccinated ewes. These results suggest that maternal vaccination with a bivalent F_{RipSc}HN/TriAdj vaccine might be an effective method to provide protection against both PIV3 and RSV in neonates.

1. Introduction

Globally human parainfluenza virus (PIV) and respiratory syncytial virus (RSV) are the most common causes of acute respiratory diseases in children under 5 years of age. On the basis of antigenic and genetic analysis PIV has been divided into four subtypes, PIV-1 to -4. PIV3 is the most pathogenic form of PIV and is associated with significant morbidity and mortality among infants and young children (Henrickson, 2003; Schmidt et al., 2011). Newborn and young infants are the populations that are most vulnerable to PIV3 infection. The highest hospitalization rate occurs in infants during the first six months of life; these infants experience fever, pneumonia and/or bronchiolitis. There still is no licensed vaccine available against PIV3. Major obstacles impeding the development of a successful PIV3 vaccine for young infants include the immaturity of their immune system and the potential interference of passively transferred maternal antibodies (MatAbs) with vaccination (Schmidt et al., 2011). Recently, maternal vaccination has been proposed as an approach to induce high levels of pathogen-specific neutralizing antibodies in pregnant women. These antibodies are transferred to the offspring and provide effective short-term protection to young infants during a period of susceptibility to pathogens, such as *Bordetella pertussis*, *Clostridium tetani* and influenza virus (Healy, 2012). Maternally derived antibodies have also been proven to be transferred passively and promote protection against respiratory viruses such as RSV (Stensballe et al., 2009).

The airway structure and function of the lungs of infants are more

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https://doi.org/10.1016/j.antiviral.2018.12.010
Received 3 August 2018; Received in revised form 11 November 2018; Accepted 11 December 2018
Available online 12 December 2018
0166-3542/

similar to those in newborn lambs than in neonatal mice. Unlike rodents, alveolar development occurs preterm in both humans and lambs. The alveoli, size of airways, capillaries, and bronchial glands are similar. Thus, this model has been used to study a wide range of human respiratory diseases such as asthma, acute lung injury, bronchopulmonary dysplasia and RSV (Derscheid and Ackermann, 2012; Scheerlinck et al., 2008). The lamb model also provides a unique opportunity to perform a maternal vaccination and neonatal challenge study. Recently, we developed a bivalent chimeric RSV-PIV3 subunit vaccine candidate consisting of a truncated version of the RSV fusion ectodomain stabilized in the pre-fusion form linked to the PIV3 HN protein formulated with a novel adjuvant (F_{RipSc}HN/TriAdj). This vaccine elicited strong antigen-specific immune responses in rodents and lambs (Garg et al., 2018).

In this study, a human PIV3 challenge model in neonatal lambs was established. Furthermore, the efficacy of F_{RipSc}HN/TriAdj-derived maternal immunity against challenge with human PIV3 was evaluated. Three-day-old lambs born to F_{RipSc}HN/TriAdj -vaccinated ewes showed significantly lower temperature response, gross pathology and PIV3 replication in the lungs than lambs born to unvaccinated ewes. These results prove that maternal immunization with a bivalent F_{RipSc}HN/TriAdj vaccine candidate confers protection against human PIV3 in neonatal lambs supporting this strategy for newborn infants.

2. Materials and methods

2.1. Cells and viruses

LLC-MK2 (ATCC, Manassas, VA, USA) and HEp-2 (ATCC) cells were maintained in minimal essential medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 0.1 mM nonessential amino acids (Thermo Fisher Scientific), 10 mM HEPES buffer (Thermo Fisher Scientific) and 50 μg/ml gentamicin (Thermo Fisher Scientific). The PIV3 C243 strain (ATCC) was propagated in LLC-MK2 cells, while the RSV A2 strain (ATCC) was grown in HEp-2 cells.

2.2. Vaccine formulation

A chimeric construct (F_{RipSc}HN), encoding the RSV F ectodomain stabilized in the pre-fusion form and linked to HN was created; the construct has a C-terminal GSGSG(H)12 tag to facilitate purification (Garg et al., 2018). F_{RipSc}HN protein was produced and purified from HEK293T cells by using His60 Ni superflow resin (Clontech, Mountain view, CA, USA) according to the manufacturer's instructions. To make the F_{RipSc}HN/TriAdj vaccine, F_{RipSc}HN protein (100 μg) was formulated with TriAdj containing poly(I:C) (250 μg) (Gibco Life Technologies, Burlington, ON, Canada), immune defense regulatory peptide (IDR) 1002 (VQRWLIVWRIRK) (Genscript, Piscataway, NJ, USA) (500 μg) and PCEP (Idaho National Laboratory, Idaho Falls, ID, USA) (250 μg) in PBS as previously described (Garg et al., 2016).

2.3. Newborn lamb as challenge model for PIV3

To investigate the susceptibility of newborn lambs to PIV3, lambs (3–4 days of age; Harry Harder farm, Clavet, SK, Canada) were randomly assigned to two groups. The first group of lambs (n = 4) was challenged intrapulmonary (IP) via a bronchoscope with 5×10^7 PFU/ml (total 5 ml) of PIV3 strain 243, and the second group (n = 2) received PBS. Clinical features of respiratory disease, including heart and respiratory rate, behavior, body weight and nasal discharge were monitored every day for five days. The lambs were euthanized, and lung samples were collected five days post challenge.

2.4. Immunization of pregnant ewes

Pregnant ewes (Suffolk sheep) were obtained from the Harry Harder farm, Clavet, SK, Canada, and randomly allocated to three groups. The first group of ewes (n = 6) was vaccinated twice intramuscularly (IM) at a four-week interval, with F_{RipSc}HN/TriAdj, the second group (n = 6) received PBS, and the third group (n = 3) was untreated. Serum samples were collected two weeks after the second immunization, around the time of lambing.

2.5. Challenge of neonatal lambs

At three to four days of age, newborn lambs born to immunized (n = 6) and non-immunized (n = 6) ewes were challenged through an intrapulmonary route via a bronchoscope with 5×10^7 PFU/ml (total 5 ml) of PIV3 strain C243. Clinical signs of respiratory disease, including heart rate, respiratory rate, nasal discharge, and body weight were recorded daily in the lambs until day 5 post challenge, when they were euthanized.

The University of Saskatchewan Animal Care Committee approved all procedures described in 2.3–2.5 in accordance with the standard policies of the Canadian Council on Animal Care.

2.6. Enzyme-linked immunosorbent assay and virus neutralization assay

PIV3 HN- and RSV F_{RipSc}-specific IgG was evaluated in serum by standard ELISA as previously described (Garg et al., 2014, 2017). Briefly, serially diluted samples were applied to HN- and F_{RipSc}-coated plates and bound antibodies were detected with alkaline phosphatase-conjugated goat anti-sheep IgG (Kirkegaard & Perry Laboratories, Gaitherburg, MD, USA). The plates were developed with p-nitrophenyl phosphate (Sigma-Aldrich Inc., St Louis, MO) substrate. PIV3- and RSV-specific neutralization titers were determined by plaque reduction assays as described previously (Garg et al., 2014, 2017). Heat-inactivated sera were mixed with PIV3 or RSV and added to LLC-MK2 cells for PIV3 and Hep2 cells for RSV. Cells were stained with 0.5% crystal violet to visualize the plaques. Results are expressed as PFU/g of lung tissue.

2.7. Postmortem examination and lung histology

Five days post challenge with PIV3, all lambs were euthanized and lungs were removed. The lungs were photographed and gross lung pathology was examined/scored on the basis of firmness of pulmonary parenchyma, pulmonary collapse, estimated percentage of lung consolidation/atelectasis, and lung weight. For lung histopathological examination, cross sections of the cranial, medial and caudal right lung lobes were fixed in formalin, trimmed and processed for hematoxylin and eosin (H&E) staining.

2.8. Virus titration

Lung tissue samples were collected on day 5 post challenge from each lobe, weighed, homogenized in a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA), clarified by centrifugation and snap-frozen in liquid nitrogen. Serial dilutions of the lung samples were added to subconfluent LLC-MK2 cells. The samples were removed after 2 h incubation and overlaid with 1.6% agarose/Eagle's Minimum Essential Medium (MEM, Gibco, Life Technologies, Burlington, ON, Canada). After five days, the overlay medium was removed, and the cells were fixed and stained with 0.5% crystal violet to visualize the plaques. Results are expressed as the average PFU/g in the lung tissue samples.

2.9. Statistical analysis

All data were analyzed using GraphPad PRISM version 7 for

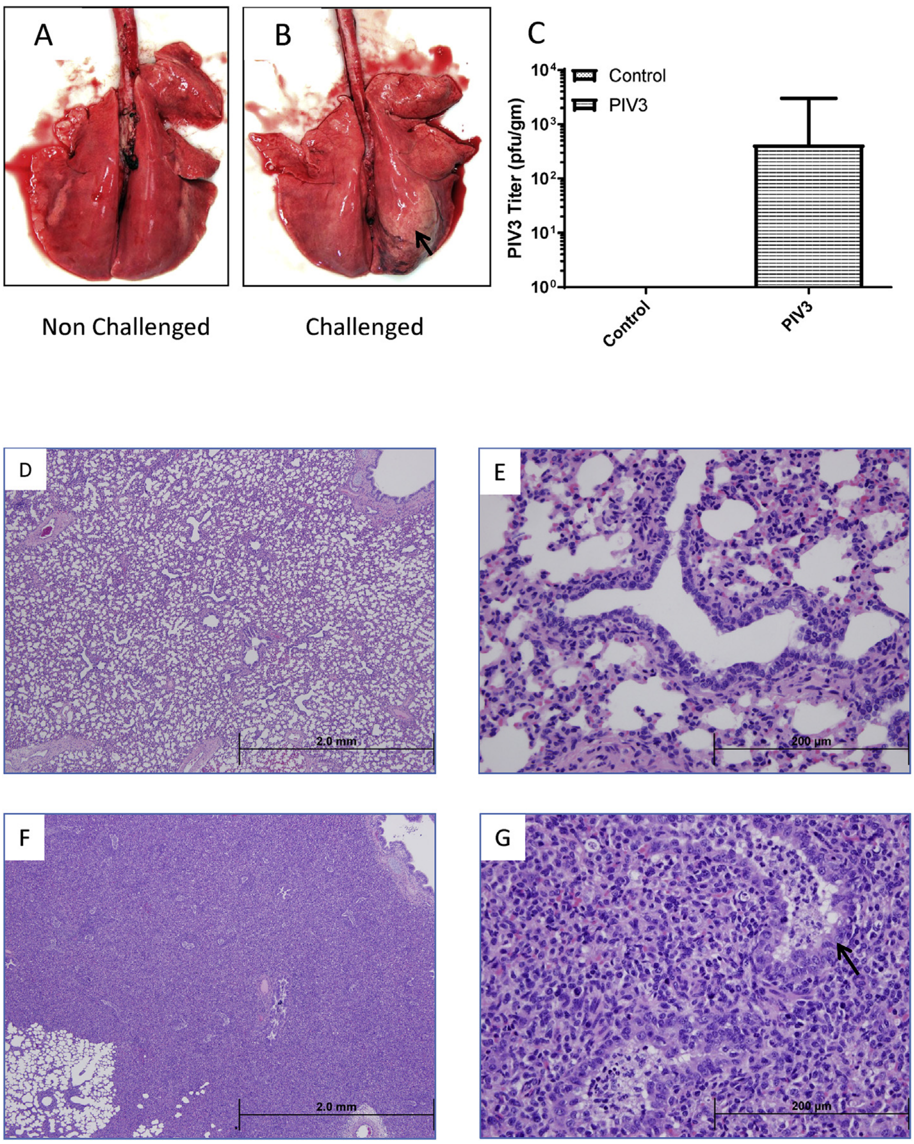

Fig. 1. Gross pathology, histopathology and virus replication in lungs from lambs challenged with human PIV3. Three to four day-old lambs were challenged with PIV3 or left untreated. Gross pathology in the lungs of one of the non-challenged (A) and PIV3-challenged (B) lambs was evaluated 5 days after challenge. (C) The virus titers were measured on day 5 after PIV3 challenge and expressed as pfu per gram of lung tissue. Representative microphotographs of lung sections of non-challenged lambs (D, E) and PIV3-challenged lambs (F, G) at 4 × (D, F) and 20 × (E, G) magnification on day 5 reveal bronchointerstitial pneumonia with bronchiolar necrosis and attenuation of the bronchiolar epithelium (black arrows in G). Bars indicate median values with interquartile ranges.

Windows (GraphPad Software, La Jolla, CA, USA). Differences among all groups were examined using one-way ANOVA, followed by a Newman-Keuls post-test. If a significant difference was found among the groups, median ranks between pairs of groups were compared by using the Mann-Whitney *U* test. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Establishment of PIV3 challenge model in newborn lambs

To establish the newborn lamb as model, three to four day-old lambs were challenged with PIV3. Clinical signs of respiratory disease, i.e. heart rate, respiratory rate and nasal discharge, were monitored every day for five days. Challenged animals did not show significant differences in clinical parameters. Five days post challenge, animals were euthanized and lungs were collected to examine viral replication. Lambs challenged with PIV3 developed gross pulmonary pathology based on several evaluation criteria including texture, consolidation and inflammation (Fig. 1Aand B); furthermore, viral replication was observed in the lungs (Fig. 1C). To confirm infection, the lungs of the PIV3-challenged animals were analyzed histologically, which showed bronchointerstitial pneumonia characterized by bronchiolar hyperplasia and attenuation with occasional bronchiolar necrosis and severe diffuse infiltration of macrophages in alveolar septae (Fig. 1Fand G).

3.2. Maternal immunization with F_{RipSc}HN/TriAdj induces production of PIV3-specific antibodies in pregnant ewes, which are efficiently transferred to newborn lambs

Maternal vaccination is a promising approach to prevent and/or reduce the severity of infectious disease in pregnant individuals and their offspring. To investigate whether the F_{RipSc}HN protein formulated with TriAdj induces production of antibodies that can efficiently transfer from mother to off-spring, pregnant ewes were vaccinated IM with F_{RipSc}HN/TriAdj prior to lambing, and the IgG levels were evaluated in both the ewes and their lambs. Significantly higher PIV3 HN- and RSV F_{RipSc}-specific antibodies were detected in serum and colostrum of vaccinated ewes (Fig. 2A–D). Furthermore, lambs born to vaccinated ewes displayed significantly higher HN- and F_{RipSc}-specific IgG levels than PBS-vaccinated animals at three to four days of age (Fig. 2Eand F).

In order to evaluate the functional quality of the antibodies in the pregnant ewes and their offspring, virus neutralization (VN) titers were determined against both PIV3 and RSV. After two vaccinations with

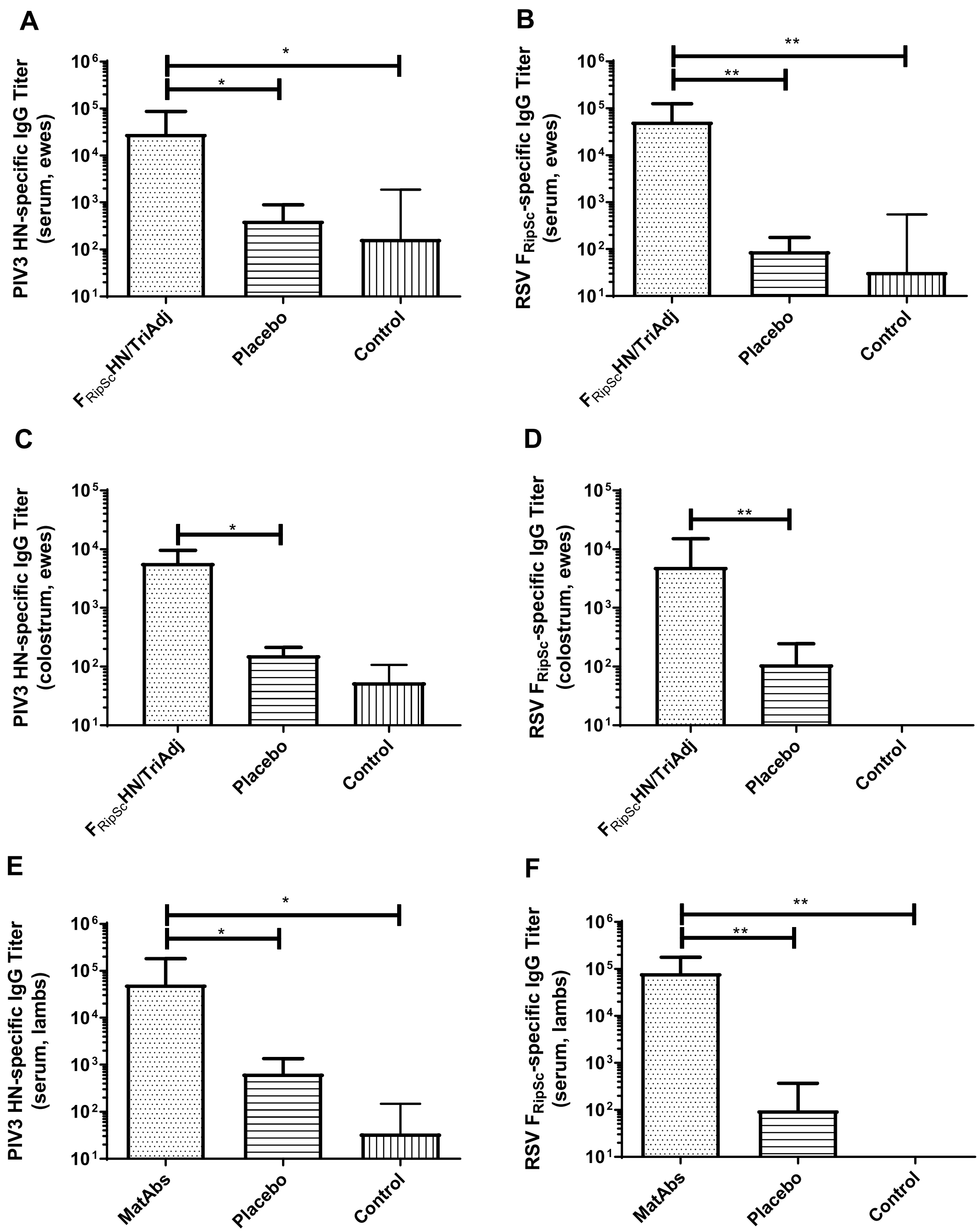

Fig. 2. Maternal immunization with F_{RipSc}HN/TriAdj induces antibodies in ewes, which are transferred to their lambs. Pregnant ewes were vaccinated twice at six and two weeks before parturition with F_{RipSc}HN formulated with TriAdj or with PBS (placebo). Serum IgG titers specific for PIV3 HN (A) and RSV F_{RipSc} (B) and colostrum IgG titers specific for PIV3 HN (C) and RSV F_{RipSc} (D) in ewes were determined two weeks after the second immunization. Serum IgG titers specific for PIV3 HN (E) and RSV F_{RipSc} (F) in lambs were determined at the time of challenge. Bars indicate median values with interquartile ranges. * $P < 0.05$; ** $P < 0.01$.

F_{RipSc}HN/TriAdj, the ewes developed high VN titers against both PIV3 and RSV (Fig. 3Aand B). At three to four days of age, just before challenge with PIV3, lambs born to F_{RipSc}HN/TriAdj-vaccinated animals had significantly higher PIV3- and RSV-specific neutralizing antibody levels when compared to lambs born to non-vaccinated ewes (Fig. 3Cand D). Overall, these results show that vaccination with F_{RipSc}HN/TriAdj produces maternal antibodies in pregnant ewes that were efficiently transferred to their offspring.

3.3. Maternal vaccination induces protective immunity from challenge with PIV3

To evaluate the level of protection induced by maternal vaccination with F_{RipSc}HN/TriAdj, the neonatal lambs born to vaccinated and non-vaccinated ewes were challenged with PIV3. After challenge with PIV3, lambs born to non-vaccinated ewes showed slightly higher respiration rate and significantly higher temperatures on day 1, which tended to remain higher till day 5 when compared to lambs born to vaccinated ewes (Fig. 4D). There were no significant differences in other clinical parameters (nasal discharge and body weight) between the groups. However, the lambs born to non-vaccinated ewes developed more

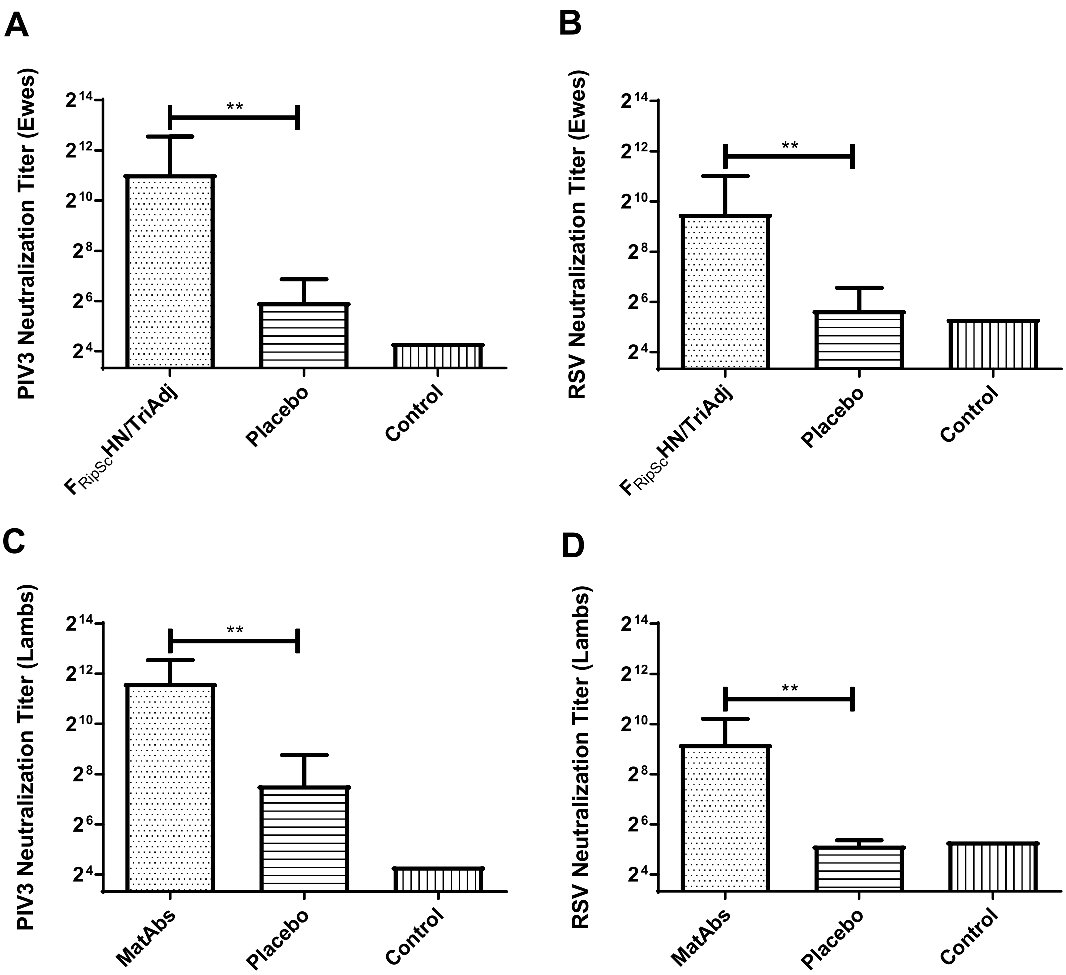

Fig. 3. Maternal immunization with F_{RipSc}HN/TriAdj promotes high levels of both PIV3 and RSV neutralizing antibodies in newborn lambs. Pregnant ewes were immunized as described in the legend for Figure 2. (A) PIV3 and (B) RSV neutralizing antibody titers in ewes after two immunizations and (C) PIV3 and (D) RSV neutralizing antibody titers in lambs at the time of challenge were determined. Virus neutralization titers are expressed as the highest dilution of serum that resulted in < 50% of cells displaying cytopathic effects. Bars indicate median values with interquartile ranges. $^{**}P < 0.01$.

severe and extensive lung pathology (Fig. 4A–C). On the basis of gross evaluation criteria such as failure of pulmonary collapse, texture, consolidation and lung weight, lambs born to vaccinated ewes displayed significantly lower gross pathology scores than lambs born to non-vaccinated ewes (Fig. 4E). Importantly, the lambs born to vaccinated ewes displayed significantly lower PIV3 titer in the lungs when compared to lambs born to non-vaccinated ewes (Fig. 4F**)**. The PIV3 titer might be partially reduced due to circulating antibodies binding to some of the virions; however, this virus is then neutralized *in vivo* and not contributing to the infection.

4. Discussion

Human PIV3 is an important pediatric respiratory pathogen, which causes serious illness and induces partially protective immunity in young infants. Neonatal vaccination is an approach that might reduce infections in the early weeks after birth, but major limitations would be the gap between vaccination and the establishment of protective immunity, as well as potential interference by MatAbs. Therefore, maternal vaccination is now considered a safe and effective strategy to provide protection against respiratory viruses such as PIV3, RSV and influenza virus within the first few months of infancy (Healy, 2012). Recently, we demonstrated that vaccination with a bivalent RSV-PIV3 vaccine candidate, F_{RipSc}HN protein formulated with TriAdj, induces strong humoral and cell-mediated immune responses in rodents and lambs (Garg et al., Antiviral Research, 2018). In the current study we evaluated the efficacy of F_{RipSc}HN/TriAdj as a maternal vaccine against PIV3 infection in neonatal lambs. We first successfully established a PIV3 challenge model in newborn lambs. This is the first report showing that neonatal lambs challenged with PIV3 display gross pathology including altered texture, consolidation and inflammation, as well as bronchointerstitial pneumonia and viral replication in the lungs similar to humans. Furthermore, we demonstrated that IM vaccination of pregnant ewes with F_{RipSc}HN/TriAdj results in efficient transfer of PIV3-specific MatAbs, which in turn mediated protection from subsequent PIV3 challenge of the newborn lambs. Previously, we showed that maternal immunization with a RSV subunit vaccine consisting of the RSV fusion protein formulated with TriAdj induces protective immunity in newborn lambs (Garg et al., 2016). These results indicate that maternal immunization with a novel bivalent RSV-PIV3 vaccine candidate, F_{RipSc}HN/TriAdj, might be a safe and effective approach to provide protection against both PIV3 and RSV in newborn and young infants.

The lamb model was used in this study because it has several advantages. Lambs are outbred animals that show pathogenesis when exposed to respiratory viruses such as RSV and PIV3, which cause similar lesions and clinical signs to those found in infants (Ackermann, 2014). Lamb lungs display close resemblance to infant lungs in their anatomy such as airway structure, alveolar development and cellularity. Furthermore, lambs have a longer neonatal period and slower maturation of the immune system than rodents, more similar to humans. Clinical studies of maternal vaccination are designed to examine

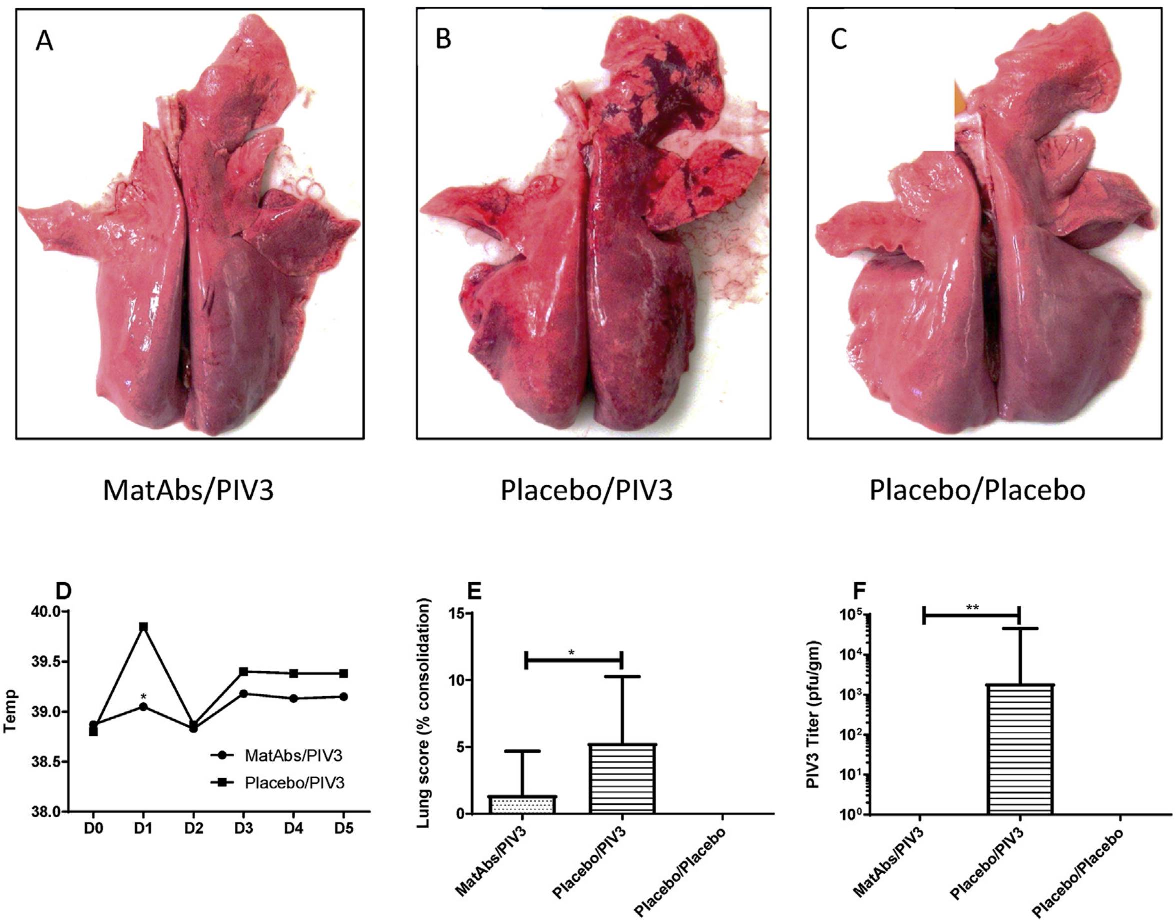

Fig. 4. Maternal immunization with F_{RipSc}HN/TriAdj reduces gross pathology and virus replication in lungs of newborn lambs challenged with PIV3. Pregnant ewes were immunized as described in the legend for Fig. 2. Lambs were challenged with PIV3 at three to four days of age or left untreated (placebo). Lungs of (A) a lamb with maternal antibodies challenged with PIV3, (B) a lamb without maternal antibodies challenged with PIV3 and (C) a placebo, unchallenged lamb. (D) temperatures; (E) gross pathology scores (percentage lung consolidation); (F) PIV3 in the lungs. Gross pathology was determined based on pulmonary collapse, firmness and extent of consolidation/atelectasis. The virus titers were measured on day 5 after PIV3 challenge and expressed as pfu per gram of lung tissue. Bars indicate median values with interquartile ranges. * $P < 0.05$; ** $P < 0.01$.

the safety and efficacy, but are not suitable to provide information on protective efficacy. In contrast, a newborn lamb model of PIV3 allowed us to assess the ability of a vaccine to induce MatAbs and protection following PIV3 challenge in neonates. In this study the MatAbs elicited by the F_{RipSc}HN/TriAdj vaccine were shown to be efficiently transferred from vaccinated ewes to their progeny, thereby providing protection against PIV3 in newborn lambs.

Very young infants (2–3 months of age) develop weak antibody responses due to poor antigen uptake, presentation and antibody affinity maturation (Lambert et al., 2014; Philbin and Levy, 2009). As a result their immune responses to vaccines are often weak, improperly polarized and/or short-lived (PrabhuDas et al., 2011). Therefore, protecting neonates from PIV3 infection by vaccination is a major challenge. Additionally, the presence of even low levels of MatAbs in infants may inhibit the efficacy of a respiratory vaccine (Crowe et al., 2001), either by reducing the antigenic load, or by masking the important B cell epitopes (Siegrist et al., 1998). These challenges can be possibly overcome by maternal vaccination to transfer high levels of PIV3-specific antibodies to the infants. This could be a safe and effective approach to protect neonates against PIV3 infection (Kaaijk et al., 2013).

Early attempts were made in the 1960s to develop a formalin-inactivated vaccine against PIV3. This vaccine was immunogenic, but failed to induce protective neutralizing antibodies or protection against natural infection (Smith et al., 1967). After the failure of formalin-inactivated vaccine, multiple PIV3 vaccine candidates, such as live attenuated mutant strains and subunit vaccines, have been developed and evaluated in various animal models with varying success (Schmidt et al., 2011). Despite decades of effort, there is still no safe and effective vaccine available for prevention of RSV or PIV3. Previously, seronegative children were vaccinated with rB/PIV3 live attenuated vaccine, which promoted high levels of PIV3-specific mucosal immunity and haemagglutination inhibition antibodies (Karron et al., 2011; Lee et al., 2001). Moreover, in another double-blind, randomized, phase-I clinical trial, a rPIV3cp45 recombinant vaccine was found to be safe, well tolerated and immunogenic after the first dose, but the booster dose was not able to induce systemic antibody responses in infants (6–12 months of age) (Karron et al., 2011). However, while live-attenuated PIV3 vaccines are immunogenic, they pose a risk of reversion to virulence, which may promote disease in young infants and immunocompromised individuals, as well as the fetus of pregnant women; in contrast, subunit vaccines are safe for all groups of individuals.

These results demonstrate that maternal immunization with an adjuvanted subunit vaccine not only elicits immune responses in the mothers, but also provides protective antibodies to the infant at birth that promote protection against PIV3 infection during the early months of life. In summary, maternal vaccination with a novel chimeric bivalent RSV-PIV3 vaccine candidate, F_{RipSc}HN/TriAdj, is a practical approach that might be effective in protecting young infants against both pediatric respiratory pathogens, PIV3 and RSV.

Acknowledgements

The authors would like to thank Wayne Connor for technical assistance, as well as Dr. Stewart Walker, Sherry Tetland and Debbie Bagnall for the handling and care of the animals. This work was supported by MOP 119473 from the Canadian Institutes of Health Research (CIHR), and the Krembil Foundation. Published as VIDO manuscript number 843.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.12.010.

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