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A single intranasal administration of virus-like particle vaccine induces an efficient protection for mice against human respiratory syncytial virus

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ABSTRACT

Human respiratory syncytial virus (RSV) is an important pediatric pathogen causing acute viral respiratory disease in infants and young children. However, no licensed vaccines are currently available. Virus-like particles (VLPs) may bring new hope to producing RSV VLP vaccine with high immunogenicity and safety. Here, we constructed the recombinants of matrix protein (M) and fusion glycoprotein (F) of RSV, respectively into a replication-deficient first-generation adenoviral vector (FGAd), which were used to co-infect Vero cells to assemble RSV VLPs successfully. The resulting VLPs showed similar immunoreactivity and function to RSV virion *in vitro*. Moreover, Th1 polarized response, and effective mucosal virus-neutralizing antibody and $CD8^+$ T-cell responses were induced by a single intranasal (i.n.) administration of RSV VLPs rather than intramuscular (i.m.) inoculation, although the comparable RSV F-specific serum IgG and long-lasting RSV-specific neutralizing antibody were detected in the mice immunized by both routes. Upon RSV challenge, VLP-immunized mice showed increased viral clearance but decreased signs of enhanced lung pathology and fewer eosinophils compared to mice immunized with formalin-inactivated RSV (FI-RSV). In addition, a single i.n. RSV VLP vaccine has the capability to induce RSV-specific long-lasting neutralizing antibody responses observable up to 15 months. Our results demonstrate that the long-term and memory immune responses in mice against RSV were induced by a single i.n. administration of RSV VLP vaccine, suggesting a successful approach of RSV VLPs as an effective and safe mucosal vaccine against RSV infection, and an applicable and qualified platform of FGAd-infected Vero cells for VLP production.

Human respiratory syncytial virus (RSV) is the most important single etiologic pathogen causing acute low respiratory illness (ALRI) in infants and young children (Schmidt et al., 2012; Esposito and Pietro, 2016), and also infects the elderly and the immunocompromised adults (Collins et al., 2007; Karron et al., 2008). However, neither any licensed vaccine available for young children, nor effective vaccine candidates are developed throughout five decades due to some disadvantages with safety, efficacy, or long-time protective responses to RSV (Power, 2008; Hall 2001; Higgins et al., 2016; Novavax Announces Topline, 2016).

The importance of RSV fusion glycoprotein (F) as a target for virus-neutralizing antibody has been recognized widely. For example, palizumab, a humanized respiratory syncytial virus monoclonal antibody specific for F protein is available for protecting high-risk children against serious complications from RSV infection (Palivizumab and a humanized, 1998). RSV F protein was also reported as the ligand for Toll-like receptor 4 (TLR4) capable of inducing proinflammatory cytokines by interaction with TLR4 and its co-receptor CD14 in epithelial cells and DCs (Kurt-Jones et al., 2000). In addition to a potential use of RSV F protein in developing an effective RSV vaccine, the matrix protein of RSV (M) has been postulated to play a critical role in virus assembly through interacting with various components of the virus and infected-cell membranes (Ghildyal et al., 2002; Henderson et al., 2002; Kiss et al., 2014). Furthermore, the highly conserved epitopes on RSV

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M protein for $CD4^+$ T and $CD8^+$ cytotoxic T lymphocytes (CTLs), especially those for CTLs, were reported to be instrumental in avoiding the enhanced respiratory disease (ERD) by facilitating viral clearance and Th1-biased immune response (Nicholas et al., 1990; Heidema et al., 2004; Rutigliano et al., 2005; Ghildyal et al., 2003, 2006; Billam et al., 2011).

As licensed vaccines against Hepatitis B virus, Hepatitis E virus and Papillomavirus, the potential applicability of VLP vaccines against RSV has emerged recently through expression of RSV VLPs in insect, yeast, avian and mammalian cells (Schmidt et al., 2014; Quan et al., 2011; Schickli et al., 2015; Qiao et al., 2016; Cimica et al., 2016; Cullen et al., 2015; Kim et al., 2015), among which the recombinants of viral antigens expressed by the mammalian cells showed the specific glycosylation and correct folding (McGinnes et al., 2011; Murawski et al., 2010; Gu et al., 2015; Walpita et al., 2015; Wu et al., 2010).

Given that VLPs provide a potential advantage to RSV vaccine in mammalian cells, we test the feasibility of viral vaccine manufacturing for human application. We constructed the recombinants of F and M proteins into first-generation adenoviral vector (FGAd), respectively. After preparing RSV VLPs by co-infecting FGAd-F and FGAd-M into a simian-derived, continuous cell line Vero, we characterized the *in vitro* function and immunoreactivity of the assembled VLPs and the ability to induce immune responses and protective immunity against RSV infection *in vivo*. We found that a single administration of RSV VLPs by intranasal (i.n.) rather than intramuscular (i.m.) route induced both powerful serum IgG responses with Th1-dominant immune type and effective mucosal immune responses. RSV challenge resulted in increased viral clearance but decreased signs of enhanced lung pathology and fewer eosinophils in VLP-immunized mice in comparison to mice immunized with FI-RSV. Our study demonstrates that the RSV VLPs were successfully assembled in Vero cells infected by FGAd-F and FGAd-M, and could serve as an effective and safe mucosal vaccine against RSV infection, suggesting that the mammalian cell expression system comprising FGAd and Vero cells is an applicable and qualified platform for VLP production.

1. Materials and methods

1.1. Preparations of recombinant adenoviruses, RSV, FI-RSV, and RSV VLPs

For recombinant adenoviruses encoding the full length RSV F and RSV M, the construction and purification were performed as reported previously (Fu et al., 2009, 2010a). Briefly, the optimized nucleotide sequences for RSV F and M were synthesized by Geneart (Regensburg, Germany) based on the GenBank database entry EF566942 and KU973192.1, and further cloned to produce FGAd shuttle plasmid of pShuttle-CMV-F and pShuttle-CMV-M, respectively, and then transfected into BJ5183 cells containing pAdEasy-1. The resulting recombinant adenoviral plasmids of pFGAd-F and pFGAd-M were linearized by *Pac*I, and transfected into and rescued in HEK293 cells separately. Large-scale preparation of FGAd-F and FGAd-M were achieved by infecting HEK293 cells in 150-mm cell culture dishes, and by CsCl (Sigma Aldrich, St. Louis, MO, USA) banding. The viral particle numbers of the prepared FGAd-F and FGAd-M were determined by the nucleic acid content.

For RSV, the preparation were performed as previously described (Zheng et al., 2012). In brief, the viruses of subgroup A RSV-Long and subgroup B RSV-BWVVR1400 (ATCC, Rockefeller, MD, USA) were propagated in HEp-2 cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS, HyClone, South Logan, UT, USA), 1% L-glutamine, 1% penicillin G/streptomycin, in a humidified incubator at 37 °C, 5% CO_2. After infected by RSV with multiplicity of infection (MOI) of 0.07 and syncytia formation, cells were scraped off the flask and centrifuged for 10 min at 1500*g*. Supernatants were pooled, filtered through a 0.45-μm sterile filter (Merck Millipore, Carolina, USA), and ultracentrifuged at 17000 rpm in P28S rotor (Hitachi, Japan) through a 10% sucrose (Sigma Aldrich) cushion for 2 h at 4 °C. The pellet was resuspended in 10% sucrose containing PBS and stored at −80 °C. The infectivity of the resulting RSV was titrated using the method of immunoenzyme assay described previously (Fu et al., 2013). Briefly, 10-fold serial dilution of RSV was prepared in virus diluents (Opti-MEM with L-glutamine containing 2% FBS, 2.5% HEPES (1 mol/L) and 1% penicillin G/streptomycin). The 100 μl of RSV dilutions was absorbed onto 85% confluency of HEp-2 cells in 96-well plate in triplicate for 60 min at room temperature (RT), and then the medium was removed and the cells were washed with DMEM without serum. Finally, DMEM containing 0.9% methyl cellulose (Sigma Aldrich) was added. After 3 days of incubation, the monolayers were fixed in 95% cold alcohol and viral replication on the monolayer was revealed by polyvalent mouse anti-RSV antibody (NCL-RSV3, Leica Biosystems, UK), incubated with horseradish peroxidase goat anti-mouse IgG (Santa Cruz Biotechnology, California, CA, USA), and visualized after adding TMB (Promega, Madison, WI, USA). The resultant RSV titers were expressed as plaque-forming units (pfu).

For FI-RSV, it was prepared as described by Kim et al. (1969). Briefly, RSV-infected cell lysates were clarified by centrifugation for 15 min at 550*g*. One part 37% formalin (Sigma Aldrich) was incubated with 4000 parts clarified lysates for 3 days at 37 °C and pelleted by ultracentrifugation for 1 h at 17000 rpm in P28S rotor. The resulting pellet was resuspended in 1/25 of the original volume in serum-free DMEM and assayed for protein concentration by BCA protein assay kit (Thermo Fisher Scientific, Inc., MA, USA).

For RSV VLPs, Vero cells, grown in 150 mm cell culture dishes up to 90% confluence, were infected with FGAd-F and FGAd-M, at 30 MOI each, for another 7 h at 37 °C, and then 30 ml of fresh DMEM with 2% FBS was added. At 24 h post infection, heparin (Merck Millipore) was added to the cells at a final concentration of 10 g/ml. Cell supernatants were collected after centrifugation at 5000*g*, 4 °C for 10 min. RSV VLPs in the supernatant were pelleted by ultracentrifugation at 22000 rpm, 4 °C for 12 h in a P28S rotor. The ensuing pellet was resuspended in NTE buffer and layered on top of a discontinuous sucrose gradient composed of 2 ml 60% sucrose, 2.5 ml 50% sucrose and 2 ml 20% sucrose (w/w). The gradients were ultracentrifuged at 35000 rpm, 4 °C for 2.5 h in a P40ST rotor (Hitachi). The fluffy layer at the 20% to 60% sucrose (w/w) interface, containing RSV VLPs, was collected and layered on 20 to 60% sucrose gradient prepared in NTE with 0.1 mol/L $MgSO_4$, and recentrifuged at 35000 rpm, 4 °C for 18 h in the P40ST rotor. Finally, the purified RSV VLPs were collected, and concentrated by ultracentrifugation in a P55ST2 rotor (Hitachi) at 34000 rpm, 4 °C for 3 h. The pellet was resuspended in NTE buffer, and the protein concentrations were assayed by BCA protein assay kit and stored at −80 °C. The content of endotoxin in the RSV VLPs was less than 0.01 EU/ml, measured using ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript Corporation, Nanjing, China), indicating the resultant RSV VLPs were qualified for preclinical studies and vaccine formulations (Dobrovolskaia et al., 2010).

1.2. Identification and characterization of RSV VLPs

For Western blot analysis, 1, 10 and 20 μg of the purified RSV VLPs were run under reducing conditions with 100 mmol/L β-mercaptoethanol (β-me, Sigma Aldrich) on sodium dodecyl sulfate 10% polyacrylamide gels. After blotting onto nitrocellulose membranes, proteins were incubated at 4 °C overnight with polyclonal