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Short Communication

Respiratory Syncytial Virus-like Particles Consisting of M, G and Prefusion F

Pramila Walpita[1]* and Lisa M Johns[1]

[1]Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A Burns School of Medicine, University of Hawaii at Monoa, USA

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***Corresponding author**

Pramila Walpita, Department of Tropical Medicine, Medical Microbiology and Pharmacology, John A Burns School of Medicine, University of Hawaii at Monoa, USA, Email: walpitap@hawaii.edu

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Abstract

Respiratory Syncytial Virus (RSV) infection is of public health concern worldwide. Globally, it is a common cause of bronchiolitis and pneumonia in childhood, outbreaks occurs every year depending on the locale. Worldwide, it is estimated that 30 million Lower Respiratory Tract Infections (LRI), 3 million hospitalization and 160,000 deaths occur annually. It also causes morbidity/mortality in the elderly/at risk adults. In spite of concentrated efforts of many over more than five decades, vaccine or therapy for this virus has remained elusive. Many conventional and newer vaccine strategies have been evaluated but none have been licensed to date. We are exploring mammalian cell-derived Virus-like Particles (VLPs) composed of the two surface glycoproteins G and prefusion F (RSV fVLPs) and M as vaccine. In prefusion F there is a neutralizing epitope at site zero that induces significantly higher Neutralizing Antibody (NtAb) titers. In preliminary studies, we have shown by Electron Microscopy (EM) that our fVLPs are functional and immunoreactive. We have done western blot with two conformation dependent antibodies AM14, and D25, and Motavizumab which is conformation independent. We have shown also that MPLA-adjuvanted fVLPs induced 8.2Log2±1.13 NtAb titers. With regular F (instead of prefusion F) lower NtAb titers are seen in cotton rats and in mice. On challenge lung virus titers in the homogenate was almost clear.

Introduction

RSV infection is of public health concern worldwide because it is a key cause of severe lower respiratory illness in infants and premature babies. According to the CDC, RSV is the "most common cause of bronchiolitis and pneumonia in children under 1 year of age in the United States". Outbreaks of disease occur every year. In children under five years worldwide, the estimated RSV disease burden is over 30 million lower respiratory tract infections, some 3 million hospitalizations, and 160,000 deaths every year [1]. In the USA alone, between 85,000 and 144,000 infants are hospitalized annually [2]. RSV is a major cause of Lower Respiratory Tract Illness (LRI) in infants and children and older adults; ~150,000 hospitalizations/year in US. It produces considerable morbidity in the elderly, and the at-risk adults such as the immunocompromised or those with cardiopulmonary disease. There is no effective treatment or prevention for RSV disease at present, and the use of passive immunoprophylaxis is limited to high risk infants.

The necessity for a vaccine for this virus has been recognized for many decades and many RSV vaccine strategies have been explored. Among them are live attenuated virus vaccines [3], subunit vaccines [4,5], replication competent as well replication defective viral vector vaccines carrying genes of interest [6,7], DNA vaccines, virosomes [8,9], nanoparticle vaccines [10,11] and others. Many Virus-like Particles (VLPs) have also been evaluated as vaccine for RSV. VLPs are recombinantly generated particles composed of multiple copies of selected proteins. Since they are composed of proteins only, do not contain the viral genome and so can't replicate, these particles are also safe. Protective efficacy of baculovirus-expressed RSV VLPs composed of RSV G or F protein, and matrix protein of influenza virus [12] and avian cell-expressed chimeric Newcastle Disease Virus (NDV) VLPs carrying the ectodomain of RSV G protein, or RSV G and F proteins have also been tested [13,14]. However in spite of concentrated efforts of so many investigators over decades, there is no licensed vaccine.

RSV vaccine development has been challenging for many reasons, among them, the young age at first infection [2], the ability of the virus to prevent the activation of a long-term adaptive immunity by the host [15,16], and the ability of RSV to evade/suppress innate immunity in multiple ways [17]. Particularly challenging has been the memory of the failed Formalin Inactivated RSV Vaccine (FI-RSV) evaluated in clinical trials in the 1960s: Tragically, the vaccinated infants who were subsequently exposed to RSV ended up developing enhanced disease rather than protection, many had to be hospitalized, and two died [18,19]. This impaired vaccine has had a negative impact on subsequent RSV vaccine development. Safety concerns rightfully persist to date [20].

Several studies have been undertaken to recognize that FI-RSV-induced immunity that triggered the enhanced disease. Studies in mice showed that the FI-RSV vaccine induced high titer but low avidity RSV-specific antibodies that failed to neutralize the virus effectively [21] and induced

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Th2 type cytokine pattern that prepared for heightened pulmonary inflammatory response on later exposure to wild type virus, [22,23]. This result was reinforced by depletion of Th2 cytokines IL-4 and IL-10 and eroded lung histopathology [24]. It has been shown since then that other non-replicating/inactivated vaccines also have the property to induce a Th2 biased response [22,25,26].

A clear understanding of the importance of appropriate Toll-like Receptor (TLR) activation [27], with reference to the FI-RSV vaccine-associated impaired immunity came more recently [28]. This study showed that poor TLR signaling resulted in low avidity antibodies and the Th2 cytokine associated enhanced disease. A UV-inactivated RSV vaccine produced a similar response. These findings were supported by the fact that in both these cases, addition of a TLR ligand such as Monophosphoryl Lipid A (MPLA) which is known to induce a Th1-biased immune response was found to alleviate these defects; both induced high affinity Neutralizing Antibody (NtAb) tiers and prevented Th2 cytokine-associated lung immunopathology. In one other study, FI-RSV vaccine formulated with MPLA resulted in mitigation of immunopathology of the lung [29]. In additional support of the importance of TLR activation, protective efficacy and absence of immunopathology has been shown in numerous non-replicating experimental RSV vaccines when they contain TLR ligands as adjuvants [8,30,31]. These findings indicate that a non-replicating/inactivated viral vaccine formulated with an appropriate TLR ligand that skews the immune response to a Th1 phenotype [32-34], would prevent immunopathology of the lung, and prove to be safe and protective.

We have used mammalian cells to make RSV fVLPs to ensure structurally authentic mammalian N- and O-glycosylation [35]. We have made the VLPs composed of two surface glycoproteins Prefusion F and G, and the matrix protein M, all retaining their native property (RSV fVLPs). We have used monoclonal antibodies AM14 and D25 that are conformation dependent antigens and Motavisumab which is conformation independent to identify the prefusion F in VLPs.

Prefusion F RSV VLPs (RSV fVLPS)

In human serum samples, most NtAb response is against prefusion F conformation. However, prefusion F has the tendency to quickly fold into a stable post-fusion form prematurely. The stabilization is achieved by modification of several residues as reported by McLellan, et al [36,37]. Prefusion F peptide induces a NtAb response and provides protection against challenge [38-40].

In a previous study in cotton rats [42] native RSV VLPs were produced with M, G and regular F, not prefusion F. MPLA/alum adjuvanted RSV VLP induced NtAb titers of around 6.5Log2 and protected the lung and nose. Protection of the nose is an important consideration because infants <6 months are obligate nose breathers; if the nose is congested, this is not a good outcome!

Materials and Methods

RSV FVLP Production

Suspension adapted HEK 293 cells (~108 cells per T75 flask) were transiently transfected with the three expression plasmids (RSV G, M and prefusion F plasmid) using Lipofectamine 2000 transfection reagent according to the manufacturer's guidelines (Invitrogen). The VLPs were harvested from the cell supernatant (SUP) at 48 hours post-transfection and were then purified as described below.

RSV fVLPs are harvested from the cell supernatant by centrifugation at 3,500 rpm for 30 minutes at 4 °C to remove cell debris and other cellular materials, and concentrated by sucrose density gradient by centrifugation. Briefly, the clarified SUPs are concentrated by ultracentrifugation through 20% sucrose cushion in endotoxin free TN buffer (0.1 M NaCl; 0.05 M Tris-HCL, pH 7.4) at 27,000 rpm (Beckman SW28 rotor) for 2-4 hours at 4 °C. The resulting VLP pellet was diluted in TN buffer, and then purified on a discontinuous sucrose gradient formed by layering 65%, 50%, 20% and 10% sucrose in TN buffer. After centrifugation at 30,000 rpm (Beckman SW41 rotor) for ~2 hours, the VLP-containing band at the interface between the 20% and 50% sucrose layers was collected, diluted in TN buffer and concentrated by ultracentrifugation for ~1 hour through a 20% sucrose cushion using SW41 rotor. The resulting pellet of purified VLPs were re-suspended in ~5% sucrose solution in TN buffer and stored at 4 °C for subsequent analysis. Cells transfected with empty pcDNA plasmid and processed similarly (referred to as "mock" particles) served as a negative control when needed.

Transmission Electron Microscopy (TEM)

In Figure 1(a), arrow points to RSV fVLP containing band in the sucrose gradient. To verify that gold-particles are functionally assembled and immune-reactive, unfixed purified VLPs were adsorbed on to glow-discharged formvar coated nickel grids (EM Sciences), stained overnight with RSV-specific polyclonal, or RSV F-specific primary antibodies diluted in buffer (1% BSA in PBS), rinsed in wash buffer (0.1% BSA in PBS), stained with appropriate colloidal gold-labeled secondary antibody (EM Sciences), washed, and then negatively stained with 2% uranyl acetate: Figure 1(b and c) shows gold-labelled particles.

Western Blot Analysis

VLP composition was determined by western blot analysis. The purified VLPs were loaded into Bolt 4-12% Bis-Tris Plus gels (Life Technologies) using 1x Bolt LDS sample buffer (Life Technologies) and were run for approximately 45min at 200V. The proteins were transferred to PVDF membrane (Life Technologies) for 3hr at 60V in 4 °C. Blots were blocked in Odyssey blocking buffer (Li-cor) for 1hr at room temperature, followed by an overnight incubation at 4 °C with anti-RSV Fusion protein monoclonal antibody (Millipore), AM14 and D25 and Motavisumab diluted 1:350 in Odyssey blocking buffer. Blots were washed four times for 5 min with PBST and then incubated for 1hr at room temperature with IR Dye 800CW Goat

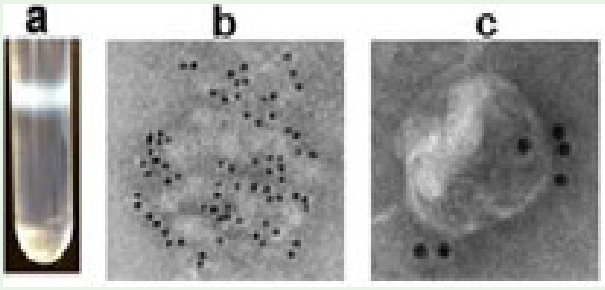

Figure 1: Show VLP containing band and immuno-reactivity of gold-labelled particles. **(a)** VLP-containing band in the sucrose density gradient. **(b) and (c)** Gold-labelled particles show that VLPs were functionally assembled and immuno-reactive.

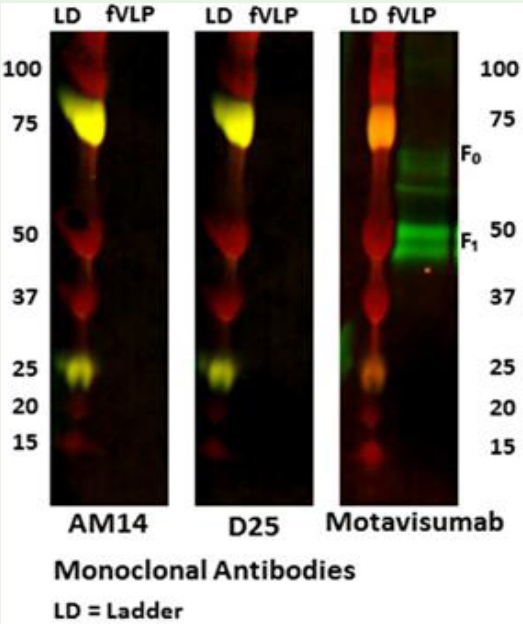

Figure 2: Western blot analysis of RSV fVLPs. Western blot analysis stained with three monoclonal antibodies, two are AM14 and D25 that are conformation (no bands) dependent and Motavizumab that is conformation independent and bands seen, F1 and F0.

anti-mouse IgG or Goat anti-Human IgG (Li-cor) diluted 1:15,000 in Odyssey blocking buffer. After washing, the proteins were visualized by scanning on Odyssey infrared imager.

Microplaque Reduction and Neutralization Assay

Vero cells were seeded into a 96-well plate at 2×10^4 cells per well and incubated overnight at 37 ˚C /5% CO_2. 2-fold serial dilutions of mouse sera were prepared in DMEM. Forty plaque forming units of RSV/A2 were added to each dilution and allowed to incubate at room temperature for 1 hour. The media was removed and cells were infected with RSV neutralization reactions. After one hour, the virus was removed and the cells were rinsed with PBS. 150ul of DMEM was added to each well prior to incubation at 37 ˚C/5% CO_2 for 48 hours. Plates were fixed with 2% Paraformaldehyde and permeabilized with

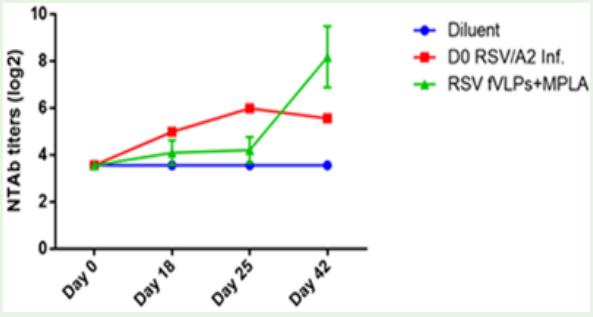

Figure 3: Neutralizing antibody response after vaccination. NtAb titers were generated after vaccination. Results: Y axis shows virus titers as Log2. X axis shows day collected. NtAb response after immunizations with two doses- On day 42 MPLA adjuvanted RSV fVLPs induced 8.2Log2. On the same day RSV titer was 5.8Log2. For RSV fVLPs +MPLA standard deviations for days 18, 25 and 42 were ±0.53, ±0.56 and ±1.03 respectively.

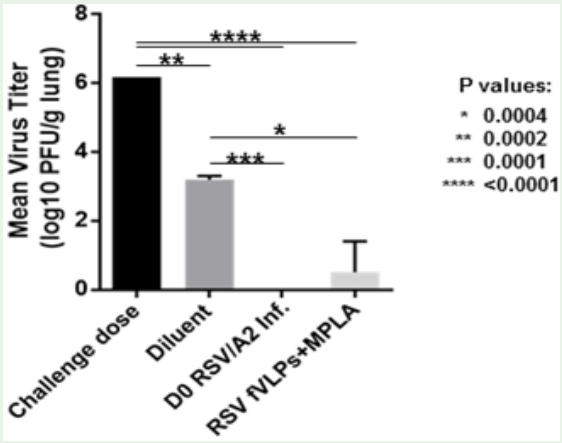

Figure 4: The effect of RSV fVLPs Vaccine on the Lung Homogenate Titers. The Y axis show mean virus titer 6log10 PFU/gram lung. The X axis is the challenge dose, diluent, D0 RSV/A2 virus, RSV fVLPs + MPLA. Results show that RSV fVLPs + MPLA; the challenge was reduced from 6log10 to near zero.

0.2% Triton. Dried plates were stored at 4 ˚C until ready for assay. Immuno-staining was done as stated in plaque assay method.

Lung Virus Titers (PFU/gm lung)

Plaques assays were performed on Vero cells (8×10^5 cells/well) in 6-well plates. The lung homogenates were tested in duplicate using 0.2ml per well. The samples were allowed to absorb for 1 hour before adding a 0.4% agarose/MEM overlay. After 7 day incubation at 37 ˚C, the overlay was removed and the cell layer was fixed with 2% paraformaldehyde followed by permeabilization with 0.2% TritonX. Plates were blocked with Odyssey Blocking buffer (LI-COR Biosciences) prior to an overnight incubation at 4 ˚C with goat anti-RSV polyclonal antibody (Millipore) diluted 1:500 in Odyssey blocking buffer. The plates were washed five times with 1x PBS/0.1% Tween 20, followed by a 1 hour incubation at room temperature with donkey anti goat IR Dye 800CW antibody (Li-cor) diluted 1:800 in Odyssey blocking buffer. Plates were then washed as above and scanned using an Odyssey Imager.

Immunization and Challenge

Six week old female BALB/c mice from Charles River Laboratories were housed at John A Burns School of Medicine, Honolulu HI. All mouse studies were approved by the Institutional Animal Care and Use Committee of the University of Hawaii. Vaccinations were given intramuscularly on day 0 and day 21. Five mice per group: Group 1 (negative control) received a diluent injection and Group 3 received 25μg of RSV fVLPs + 15μg MPLA. Group 2 (gold standard): RSV A2 at 10^6 PFU was Intra-Nasally (IN) inoculated on day 0. Day 42 was the challenge day; RSV A2 at 10^6 PFU was instilled IN for group 1 and Group 3. Harvest was day 46. Blood was collected by submandibular bleeding before vaccinations on day 0, and on days 21, 42 and 46; Lungs were also collected.

Results

In Figure 1(a), VLP-containing band in the sucrose density gradient is shown. In Figure 1(b and c), gold-labelled particles show that VLPs are functionally assembled and immuno-reactive. Western

blot of RSV fVLPS are shown in Figure 2. Monoclonal antibodies AM14 and D25 show no bands because they are conformational dependent. However, Motavisumab is conformational independent and do show bands F0 and F1, indicating that the prefusion F is included in the VLPs.

After vaccination, NtAb antibody response was determined as described in Materials and Methods, NtAb response after two dose immunization (Figure 3). On day 42 MPLA adjuvanted RSV fVLPs induced 8.2Log2. On the same day RSV/A2 generated titer was 5.8Log2 much lower than that generated by adjuvanted VLPs. The effect of RSV fVLPs Vaccine on the Lung Homogenate Titers is shown in Figure 4. After RSV/A2 virus challenge, RSV fVLPs + MPLA immunized mice show that the challenged virus was reduced from 6log10 to near zero.

Discussion

The RSV fVLPs are composed of G, prefusion F, and M proteins. Prefusion F has neutralizing epitope at site zero that induces higher NtAb than the regular F protein. We have included both the G and prefusion F proteins in our VLPs. for optimal immune response since together they have many T cell epitopes and all antibody neutralizing epitopes between them [42,16]. Clearly the presence of F fragment is crucial for vaccine development. We have shown by immuno-gold labelling that the RSV fVLPs were functionally assembled and immuno-reactive. We have shown western blot analysis also. Monoclonal antibodies AM14 and D25 are conformational dependent and show no bands. However, Motavisumab is conformational independent and do show bands indicating that prefusion F is present in the VLPs. We have also shown that RSV fVLPs induce significantly higher NtAb titers of 8.2Log2±1.3 and provide better protection than some of the previous studies using regular F as in RSV VLPs: NtAb titers were much lower in cotton rats ~6.5Log2±0.5 [42]and in mice ~6Log2 [14]. We have also done virus titers in homogenized lung. Challenge titer was 6Log10, whereas titer of MPLA adjuvanted RSV fVLPs was reduced to near zero, indicating the lung was fully protected.

We have chosen a TLR 4 ligand MPLA by design, because it induces a Th1 biased response [33]. TLR-4 is the only TLR that can use all the four TIR-containing adaptor molecules, namely TRIF, TIRAP, MyD88, and TRAM, leading to two different downstream signaling pathways, (a) TRIF-dependent, MyD88 independent pathway that leads to the activation of transacting factor IRF-3 with delayed NFkB production, and (b) MyD88-dependent pathway which results in early robust activation of NFkB [43,44]. Importance of TLR-4 activation is further emphasized by the fact that co-administration of inactivated vaccines like FI-RSV with MPLA is known to result in mitigation of Th2 cytokine-associated ERD [29]. Furthermore, ERD is not seen in non-replicating vaccines formulated with MPLA [8,41].

In conclusion we have shown in this preliminary study, that high NtAb titer of 8.2Log2 reduced the lung challenge from 6Log10 to near zero; in other words the lung was almost clear of the virus. We will continue to evaluate RSV fVLPs by studying histopathology of the lung in animals and Th1/Th2 biased response.

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