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3 **TITLE**4 **A Novel Respiratory Syncytial Virus-Like Particle (VLP) Vaccine Composed of the
5 Postfusion and Prefusion Conformations of the F Glycoprotein**

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7 Velasco Cimica¹, Hélène Boigard¹, Bipin Bhatia², John T. Fallon³, Alexandra Alimova⁴, Paul
8 Gottlieb⁴, Jose M. Galarza^{1#}

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10 ¹ TechnoVax, Inc. 765 Old Saw Mill River Road, Tarrytown, NY 10591, USA.11 ² EMD-Millipore Corporation, 290 Concord Road, Billerica, MA 02013, USA.12 ³ Department of Pathology, New York Medical College, Westchester Medical Center, Valhalla,
13 NY10595, USA.14 ⁴ Department of Pathobiology, Sophie Davis School of Biomedical Education, City College of
15 New York, 160 Convent Avenue, New York, NY 10031, USA.16 # Corresponding Author: jmgalarza@technovax.com

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18 **RUNNING TITLE:**19 **A Respiratory Syncytial Virus-Like Particle Vaccine**

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25 **ABSTRACT**

26 Respiratory Syncytial Virus (RSV) is the leading cause of severe respiratory disease in
27 infants and children and represents an important health burden for the elderly and the
28 immunocompromised globally. In spite of decades of research efforts, no licensed vaccine is
29 available for RSV.

30 We have developed virus-like particle (VLP) based RSV vaccines assembled with the
31 human metapneumovirus matrix protein (hMPV M) as the structural scaffold and the RSV fusion
32 glycoprotein (F) in either the postfusion or prefusion conformations as its prime surface
33 immunogen. Vaccines were composed of postfusion F, prefusion F or a combination of both
34 conformations, and formulated with the squalene-based oil emulsion as adjuvant. Immunization
35 with these VLP vaccines afforded full protection against RSV infection and prevented detectable
36 viral replication in the mouse lung after challenge. Analysis of lung cytokines and chemokines
37 showed that VLP vaccination mostly induced production of IFN- γ , marker of Th1-mediated
38 immune response, which is predominantly required for viral protection. Conversely,
39 immunization with a formalin inactivated RSV (FI-RSV) vaccine induced high levels of
40 inflammatory chemokines and cytokines of the Th2 and Th17 types of immune mediated
41 responses, as well as severe lung inflammation and histopathology. The VLP vaccines showed
42 restricted production of these immune mediators and did not induce severe bronchiolitis or
43 perivascular infiltration as seen with the FI-RSV vaccine. Remarkably, analysis of the serum
44 from immunized mice showed that the VLP vaccine formulated using a combination of
45 postfusion and prefusion F elicited the highest level of neutralizing antibody and enhanced the
46 Th1-mediated immune response.

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49 INTRODUCTION

50 Human Respiratory Syncytial Virus (RSV) is the leading cause of severe pediatric
51 pulmonary disease worldwide. RSV infects nearly all infants at least once by the age of 2 years.
52 Epidemiological studies around the globe indicate that 2-5% of the children infected with RSV
53 require hospitalization with the most severe morbidity and mortality disproportionality affecting
54 premature infants. RSV disease causes 100,000 to 200,000 fatalities per year globally (1, 2). It is
55 believed that severe RSV infection can predispose children to develop wheezing with future
56 illnesses and potentially asthma (3, 4). RSV infection elicits neutralizing antibodies and a T-cell
57 response that wanes over time, consequently the patient is often unprotected against reinfection
58 (5, 6). Furthermore, elderly people show a greater risk of severe RSV disease upon reinfection
59 (7). Despite decades of research efforts, no licensed vaccine is currently available to control or
60 prevent RSV infection (8). Vaccinology research shows that the F glycoprotein is the most
61 attractive target for eliciting neutralizing antibodies against the virus. RSV displays different
62 conformations of F that are antigenically distinct: the highly stable postfusion, and the metastable
63 prefusion (9). Magro et. al. (10) have demonstrated that antibodies specific to prefusion F
64 account for most of the neutralizing activity in a prophylactic human Ig preparation and
65 immunized rabbits. Subsequently, McLellan and coworkers (9) determined the protein structure
66 of the prefusion F by X-ray crystallography and identified the prefusion-only antigenic site ϕ
67 (Fig. 1A). While Palivizumab can recognize both postfusion and prefusion structures, a subset of
68 highly neutralizing antibodies 5C4, AM22 and D25 bind specifically to the prefusion antigenic
69 site ϕ (9, 10). Interestingly, AM14 and MPE8 neutralizing antibodies are also able to very
70 efficiently recognize the prefusion F using alternative antigenic sites. This demonstrates that the

71 prefusion F expresses multiple epitopes suitable for target therapy (11, 12), which are not
72 exhibited in the postfusion conformation.

73 Adopting structural vaccinology, our group has developed virus-like particle (VLP)
74 vaccines containing recombinant postfusion and prefusion F hybrids together with the human
75 metapneumovirus (hMPV) matrix protein (M). Efficacy studies showed that immunization with
76 prefusion F VLP, postfusion F VLP or a combination of both, afforded complete protection
77 against an RSV virus challenge. Importantly, VLP vaccination was safe and effective in
78 stimulating a Th1 type cytokine profile and the combo VLP vaccine elicited the highest-level of
79 IgG2a antibody and neutralization activity.

80

81 MATERIALS & METHODS

82

83 Structural Vaccinology

84 The RSV fusion (F) (GenBank: ACO83302.1) and human metapneumovirus matrix (M)
85 (GenBank: AIY25728.1) genes were codon optimized and chemically synthesized (Blue Heron
86 Biotech, WA). Prefusion F mutants were designed by protein structure analysis using the Cn3D
87 software (NCBI, MD) and data from NCBI repository (9, 13). Wild type optimized RSV F was
88 subcloned into an expression vector and mutagenized by cysteine substitutions using the
89 QuickChange II kit (Agilent, CA) and DNA oligos (IDT, TX). The cytoplasmic tail (CT) and the
90 transmembrane domain (TM) of RSV F was swapped with HMPV F analogs domains using
91 recombinant DNA methods: hRSV F sequence from amino acids 1-524 (GenBank:
92 ACO83302.1) was joined to the hMPV F sequence from amino acids 489-539 (GenBank:
93 AEK26895.1). Constructs were fully sequenced for quality verification.

94

95 Vaccine

96 RSV virus-like particles (VLPs) were produced in a suspension culture of mammalian
97 cells following transient transfection of the VLP protein expression plasmids. Expi293F cells
98 were maintained in serum free Expi293 expression medium (Life Technologies, CA).
99 Transfection reaction was assembled in separate tubes: 1.0 µg DNA vectors, 3.0 µg of 25 kDa
100 linear polyethylenimine (PEI) (Polysciences, PA), and 0.1 ml OptiMem, for each 1ml of cells
101 culture (2.5×10^6 cells/ml). The plasmid for RSV F and HMPV M were transfected at a ratio of
102 70% and 30% respectively. The mixture was incubated for 15 min at room temperature and then
103 added to the cells. Forty-eight hours after transfection, the culture supernatant was clarified by
104 centrifugation at 8,000 x g for 10 min. and VLPs concentrated by ultracentrifugation for 2 hours
105 at 140,000 x g using a SW 28 rotor (Beckman Coulter CA). Pellets were resuspended in 250 µl
106 of buffer E (250 mM Sucrose, 100 mM MgSO₄, 10 mM TRIS pH 7.5) containing protease
107 inhibitors (Thermo Scientific, MA). The VLPs were subsequently purified by ultracentrifugation
108 through a continuous sucrose gradient (10%-50%) for 4 hours at 180,000 x g using a SW 40Ti
109 rotor. The VLPs were collected from the 15%-25% fraction and further purified by ultrafiltration
110 using Vivaspin system 100kDa cut-off (Sartorius, NY). The VLPs were characterized by
111 Western blot, dot blot, and electron microscopy. The VLP combo vaccine was prepared by
112 blending equal amounts (2 µg of F content) of VLPs with prefusion F and postfusion F per dose
113 (4 µg total F content). FI-RSV vaccine was produced using the protocol described by Prince et
114 al. (14). The final preparation was adjuvanted with Imject Alum (Thermo Scientific, MA) to a
115 final concentration of 4 mg/ml of Aluminium Hydroxyde and then diluted 1:25 in PBS for
116 animal administration.

117

118

119 Dot Blot and Western Blot

120 The immune reactivity and antigen concentration of the VLP vaccine was assayed using a
121 dot blot assay. VLP samples in a volume of 1-5 µl were absorbed into nitrocellulose for 15 min
122 and then blocked for 1hr with 3% non-fat dry milk in Tris-buffered saline plus 0.1% Tween-20
123 (TBS-Tween). Primary antibodies were: Palivizumab (Synagis®, MedImmune, MD), 131-2a
124 (EMD-Millipore, MA), 5C4 (9) or anti-hMVP M (GeneTex clone 4821, CA). Horseradish
125 peroxidase (HRP) conjugated mouse anti-human antibody, or goat anti-mouse antibody were
126 used as secondary antibody. Detection was performed using ECL substrate and digital imaging
127 system (FluorChem M System, ProteinSimple CA). The dot blot with Palivizumab was used for
128 quantifying F content in the vaccines as compared to a serial dilution of recombinant RSV F
129 protein standard (Sino Biological, PA) and quantified using AlphaView SA imaging software
130 (ProteinSimple, CA). Western blot was performed using Novex gel system (Life Technologies,
131 CA) and Nupage 4-12% Bis-Tris Gel (Life Technologies, CA).

132

133 Electron Microscopy Analysis of the VLP particles

134 The VLP particles were examined by negative staining and electron microscopy (Zeiss
135 902 TEM, U=80kV). 5 µl of resuspended particles were applied to a formvar coated copper grid
136 FCF300-Cu (Electron Microscopy Science, PA) and stained with 2.0% of phosphotungstic acid,
137 pH 7.0. Immunogold labeling was performed as follows: 5 µl of resuspended samples were
138 applied to formvar-coated grids and incubated for 5 min at room temperature (RT). The grid was
139 then washed 5 times with buffer (0.1% FBS in PBS, 10 mM glycine, 0.01% NaN₃); fixed with
140 4% paraformaldehyde in PBS for 15 min, and blocked in 1% BSA in PBS for 30 min. The
141 sample was floated onto 40 µl of Palivizumab (Synagis, MedImmune MD) at 1:1000 dilution in

142 0.1% BSA in PBS + 0.01% of NaN₃ buffer overnight at 4°C. The grid was then placed in a
143 humid chamber to prevent evaporation. Following 5 washes with buffer, the grid was placed on a
144 drop of 6 nm gold beads conjugated with goat anti-human polyclonal IgG antibody, (Abcam,
145 MA), 1:3 dilution in 0.1% BSA in PBS + 0.01% of NaN₃ for 2 hours at RT. Then sample was
146 washed 5 times in washing buffer and stained with 2.0% of phosphotungstic acid, pH 7.0.

147

148 **Virus Production and Titration**

149 This study was performed in compliance of BSL-2 regulations. The RSV A2 strain was
150 obtained from the ATCC-VR-1540 (ATCC, VA) and used for murine challenge, production of
151 FI-RSV vaccine, immunization and viral assays. RSV was propagated in HEp-2 cells (ATCC
152 VA, CCL-23) and using culture-medium DMEM with 2% fetal bovine serum (FBS) (Life
153 Technologies CA) according to the supplier protocol. Viral titration was performed by plaque
154 assay using HEp-2 monolayer. HEp-2 cells were infected for 1-hour for viral absorption with 10-
155 fold serially diluted virus in serum free DMEM medium, from 10⁴ to 10⁸ dilution range.
156 Subsequently, an overlay of 1% methylcellulose (Sigma-Aldrich MO, C-4888) in DMEM
157 medium supplemented with 2% FBS was applied to each well to prevent viral particles diffusion.
158 After 4 to 5 days of incubation the overlay was removed and cells were fixed using cold
159 methanol for 20 min, at -20 °C. Virus plaques were stained by immunocytochemistry techniques
160 using an anti-RSV goat antibody (EMD-Millipore Corporation CA, AB1128) diluted 1:500 in
161 blocking buffer as a primary antibody. Immune detection was performed using HRP conjugated
162 rabbit anti-goat antibody (Abcam MA, ab97105) as a secondary antibody. Immunostaining was
163 developed using DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories CA, SK-4100) and
164 plaques were counted using a light microscope with 4X to 20X objective magnification.
165

166

167 **Murine Model for immunization and RSV infection**

168 BALB/c mice (*mus musculus*) 6-week-old females from Charles River were housed at the
169 Department of Comparative Medicine, New York Medical College, Valhalla, NY. Mice were
170 anesthetized with Ketamine (100 mg/kg)/Xylazine (10 mg/kg) administered via intraperitoneal
171 injection before immunization or blood collection. Mice were immunized by intramuscular (IM)
172 injection with 50 µl of postfusion, prefusion or the combo VLP vaccines as well as FI-RSV, and
173 placebo control (n = 10 per group). Immunizations were administered at day 1 and 14 and each
174 VLP vaccine dose contained 4 µg of total recombinant RSV F admixed in a 1:1 volume with a
175 squalene-based oil-in-water nano-emulsion AddaVax (InvivoGen, CA). The placebo group
176 received PBS admixed with AddaVax at 1:1 volume. Serum was collected by retro-orbital
177 bleeding before and after immunization. Mice were challenged with 1×10^6 pfu of RSV A2 strain
178 contained in a 50 µl administrated via the intranasal route as small drops (Pipetman with
179 ultraslim tip) at day 28. Group of mice were sacrificed at 4, and 7 days post challenge for lung
180 and blood harvest (S. Fig. 2).

181

182 **Pulmonary RSV Quantification by Plaque Assay**

183 Lungs from RSV infected mice were harvested, weighed, and homogenized using an
184 Omni tissue homogenizer (Omni International) in Opti-MEM I media containing 25% sucrose,
185 penicillin-streptomycin-glutamine (Life Technologies, CA), and 2.5 µg/ml Fungizone (Chem-
186 Impex International Inc., IL). Lung supernatants were obtained by centrifugation and viral titer
187 measured by plaque assay as above.

188

189

190 Neutralization Assay

191 Plaque reduction neutralization assays (PRNT) were performed in duplicate using serum
192 samples collected before viral challenge (day 28). Serial dilution of serum was incubated with
193 100 pfu of RSV A2 virus for 1 hour at 37 °C and neutralization power measured by a plaque
194 assay in HEp-2 as described above. IC₅₀ calculation was performed applying the Probit analysis
195 (15).

196

197 Luminex Cytokines and Chemokines Analysis

198 Magnetic bead-based sandwich immunoassays for cytokines using MILLIPLEX MAP
199 multiplex Mouse Cytokine Panel 1 (EMD-Millipore, MA) were performed according to the
200 manufacturer's instruction. Lung samples (25 µl) were analyzed in duplicate wells using a
201 Luminex MagPix (Luminex Corp., TX). Cytokine concentrations were determined by Luminex
202 Xponent 4.2 and EMD-Millipore Milliplex Analyst v5.1 using 5-p log analysis. IFNγ analysis in
203 lung fluids was confirmed using an ELISA Kit (eBioscience, CA).

204

205 ELISA Analysis of IgG Subtypes

206 Each well of ELISA assay plates (Corning Costar NY, 3912) was coated with RSV A2
207 strain containing 100 ng of F protein content determined by dot blot analysis using purified
208 recombinant F protein (Sino Biological, PA) as standard, and incubated at 4 °C overnight (see
209 above). Serum samples were serially diluted in blocking buffer (5% milk in TBS-tween), applied
210 in triplicated to the ELISA plates and incubated for 2 hours at room temperature. Following
211 washes, detection was carried out using the following antibodies: IgG1 subtype (Jackson
212 ImmunoResearch Lab. PA, 115-035-205), IgG2a subtype (Jackson ImmunoResearch Lab. PA,

213 115-035-206). The ELISA measurements were performed using chemiluminescence (ECL)
214 method and microplate reader (BioTek VT, Synergy H1). Endpoint calculation for ELISA assay
215 was performed according to Frey et. al. (16).

216

217 **Histopathology**

218 Lungs were harvested on day 4 post-infection and fixed in 10% buffered formalin
219 phosphate. Lung samples were processed at the Department of Pathology at the New York
220 Medical College (Valhalla, NY) and stained with hematoxylin and eosin (H&E) following a
221 standard protocol (17). Examination and scoring of lung histopathology was performed by blind
222 evaluation of the H&E slides.

223

224 **Statistics**

225 Data was statistically analyzed and graphed using GraphPad Prism (GraphPad Software
226 CA), and errors bars are representing calculated standard error. Statistical significance of the data
227 was measured by one-way ANOVA test with Dunnett's multiple comparisons between
228 experimental conditions, and t-test. Analysis of the ratio of IgG2a versus IgG1 was achieved
229 using Taylor expansion statistical approach for calculating standard errors. Pictures and images
230 were represented using Adobe Photoshop (Adobe, CA).

231

232 **RESULTS**

233

234 **Development of recombinant RSV F exhibiting postfusion and prefusion conformations**

235 Structural analysis of the postfusion F shows that the C-terminus of F2 is in the opposite
236 orientation and distant from the N-terminus of F1 (Fig. 1B), whereas these domains are adjacent

in the prefusion conformation (9, 13). We identified within this region several amino acids that are in close proximity separated by less than 10 Angstroms. Based on this analysis, we generated 9 recombinant constructs with alternative disulfide bonds between these domains to stabilize F in its prefusion conformation and one with the furin cleavage site mutated (S. Table 1). Prefusion F VLPs were produced in mammalian cells and analyzed by dot blot with the mAbs Palivizumab (antigenic site II) to measure F protein expression, and 5C4 to assess the presence and stability of the antigenic site ϕ (S. Table 1) (S. Fig. 1). We evaluated the postfusion state with the mAb 131-2a which is specific for the antigenic site I (S. Table 1). We found that the most stable prefusion F contained an intra-chain disulfide bond inside the F1 subunit described by McLellan et. al. (S155C/S290C) (18), plus an inter-chain disulfide bond between the F1 and F2 subunits with the cysteine substitutions: A102C and I148C (Fig. 1A and 1B). By dot blot analysis with 5C4, we found that the prefusion F recombinant is recognized 19.2 +/- 2.4 fold more than the postfusion F (Fig. 1C). To assess whether the disulfide bridge A102C/I148C enhanced the stability of F prefusion, we tested an intermediate mutant having only one cysteine change, A102C. Indeed the mutant A102C construct demonstrated reactivity with 5C4 equivalent to wild type F postfusion construct. In addition, dot blot analysis demonstrated that the combination of disulfide bonds S155C/S290C with A102C/I148C enhances 5C4 reactivity with respect to the single disulfide bond constructs (S. Fig. 1). VLPs assembled with this mutant (S155C/S290C plus A102C/I148C) were used in the vaccine studies. In addition, this F construct contains the cytoplasmic tail domain of HMPV F (Fig.1A), which seems to further stabilize the structure of F incorporated in the particles as reflected by strong reactivity with 5C4 and Palivizumab (Fig.1C). This and other mutants without the HMPV F tail demonstrated strong reactivity with 5C4 but weaker reactivity with Palivizumab (S. Table 1).

260 Generation of VLPs displaying RSV F postfusion or prefusion conformations

261 The RSV envelope displays three virally encoded and membrane anchored proteins F, G
262 and SH (Fig. 2A) (19). Underlying the envelope resides the matrix (M) protein, which during
263 morphogenesis multimerizes and drives virion assembly and budding (20). To assemble VLPs,
264 we utilized the RSV F either postfusion or prefusion together with the matrix protein (M) of the
265 human metapneumovirus (hMPV) as scaffold, which as we found is more efficient than the RSV
266 M in VLP formation (data not shown). To optimize the interaction of RSV F and hMPV M, we
267 replaced the cytoplasmic domain of the RSV F protein with the analogous domain of the hMPV
268 F protein, which based on yield analysis in comparison to unmodified F demonstrated to enhance
269 RSV F recruitment and incorporation onto the VLP surface (Fig. 1C).

270 Analysis of purified VLPs by Western blot showed that the RSV F hybrid co-purified with the
271 hMPV M (Fig. 2B) and that replacement of the cytoplasmic tail enhanced incorporation of the
272 RSV F into particles as compared to wild type RSV F (data not shown). These results suggest
273 that the RSV F hybrids interact with the hMPV M via its engineered cytoplasmic domain.

274 Examination of purified VLPs by electron microscopy (EM) showed spherical structures of
275 ~80nm in diameter that display F spikes protruding from the membrane envelope (Fig. 2C and
276 D). Immuno-gold labeling EM confirmed that the spikes were indeed composed of the
277 glycoprotein F (Fig. 2D)

278

279 Efficacy evaluation of RSV F VLP vaccines in a murine model

280 To assess the VLP vaccines protective efficacy, BALB/c mice were immunized twice
281 with formulations containing either i) postfusion F VLPs, ii) prefusion F VLP, or iii) a
282 combination of both VLPs (combo), and then challenged with RSV (S. Fig. 2). To evaluate the

283 safety of the VLP vaccines, we included a group of mice immunized with the FI-RSV vaccine
284 expected to induce vaccine-enhanced disease. Analysis of protective efficacy on day 4 post-
285 challenge showed that the VLP immunized mice were completely protected from RSV
286 replication and did not show a detectable viral load (<50 pfu/gram of lung tissue), whereas the
287 placebo group demonstrated high levels of infective particles inside their lungs (75,000 pfu/gram
288 of lung tissue) (Fig. 3). Assessment of viral load at day 7 post-challenge demonstrated the
289 absence of replicating virus in the lungs of all the animals (data not shown). To appraise the
290 quality and magnitude of the antibody response, we measured serum-neutralizing activity of
291 immunized animals prior to viral challenge (day 28) by plaque reduction neutralization test
292 (PRNT) (Fig. 3B). This analysis showed that the combo VLP vaccine (postfusion plus prefusion
293 F) elicited the highest level of neutralizing antibodies as compared to either the postfusion or
294 prefusion F single vaccine formulations. The prefusion F VLP vaccine, however, elicited higher
295 neutralizing antibody titers than the postfusion, results that agree with previous reports (21, 22).
296 On the other hand, the FI-RSV vaccine failed to induce an appreciable level of neutralizing
297 antibodies. The Palivizumab control demonstrated an IC₅₀ neutralizing activity of 2 µg/ml, a
298 value that is similar to previous determinations (23).

299

300 **VLP vaccine stimulates a balanced IgG response**

301 We assessed the magnitude of the IgG2a and IgG1 serum responses, which are correlates
302 of Th1 and Th2 development respectively (Fig. 3C and 3D). Mice that received VLP vaccine
303 demonstrated induction of robust serum IgG responses as compared to the preimmune samples.
304 On the other hand, serum from FI-RSV immunized mice showed the highest level of IgGs
305 induction suggesting that antibodies toward multiple viral proteins were produced and detected

306 in the whole virus ELISA. Analysis of IgG subtype demonstrated that the combo vaccine elicited
307 a balanced Th1- versus Th2-mediated response, associated with a greater IgG2a versus IgG1
308 ratio (Fig. 3D). As expected, placebo control demonstrated background levels of total and
309 specific IgGs against RSV.

310

311 **Analysis of the cytokine profile in VLP vaccinated and control mice after RSV infection**

312 We applied Luminex technology to study the cytokine and chemokine levels in lung
313 homogenates of VLP vaccinated and control mice four days after challenge. We evaluated
314 cytokine markers that correlate with Th1, Th2, and the Th17 type of immune responses as well
315 as IL-10 (Fig. 4A, 4B, 4C and 4D). VLP immunized mice showed a robust IFN- γ response in
316 comparison to the placebo control (Fig. 4A). On the other hand, immunization with the FI-RSV
317 vaccine stimulated a strong cytokines response that qualitatively and quantitatively differed from
318 the one elicited in mice immunized with the VLP vaccine or placebo. We found that FI-RSV
319 immunization induced high levels of the cytokines IFN γ , TNF α , IL-4, IL-10, IL-17 and IL-1 β all
320 of which have been associated with the exacerbation of RSV disease (24–29) (Fig. 4A, 4B, 4C
321 and 4D). In contrast, multiplex analysis of placebo control demonstrated very low expression of
322 these cytokines indicating that RSV replication did not trigger or perhaps curtailed production of
323 these immune signaling molecules (Fig. 4A, 4B, 4C, and 4D). This is in agreement with Lambert
324 and coworkers (30), who also found very low levels of IFN γ , IL-5, IL-13 and IL-17 in the lung
325 of the placebo group at day 4 post challenge. However, higher doses of challenging virus (e.g.
326 1×10^7 PFU) increases the level of secreted cytokines as described by Rutigliano et. al. (29).
327 Chemokines recruit inflammatory cells to the infected tissue and are particularly elevated in
328 bronchiolitis (31, 32). Thus, we analyzed Eotaxin, MCP-1, MIP-1 α , and RANTES, all of which

329 are involved in lung immune cell infiltration during bronchiolitis (Fig. 5). While FI-RSV
330 vaccine strongly augmented each chemokine, the VLP immunized animals had a less significant
331 induction of these inflammatory mediators, which were closer to that seen in the placebo control.

332

333 **Evaluation of VLP vaccine safety by lung histopathology examination**

334 VLP vaccine safety and tolerability was further evaluated by histological examination of
335 lung tissue of VLP vaccinated mice after 4 days postinfection (Fig. 6A and B). Placebo control
336 mice that received a primary infection with 10^6 pfu of RSV experienced some interstitial cellular
337 infiltrate but limited or no sign of perivascular infiltration at day 4 post-challenge, indicating that
338 virus replication was tolerated without provoking serious lesions. Indeed, a previous report (30)
339 has shown minimal eosinophilic infiltration in primary infected mice under the same
340 experimental conditions.

341 FI-RSV vaccinated mice displayed a massive perivascular, peribronchial and interstitial
342 infiltration of inflammatory cells. In contrast, the VLP immunization showed a limited immune
343 cell infiltration. Blinded scoring of perivascular infiltration demonstrated that the combo VLP
344 vaccine had the lowest level of histological changes (Fig. 6 B). The perivascular infiltration in
345 the postfusion and prefusion vaccine groups was more pronounced than that of the placebo
346 control; however the overall lung architecture was not significantly different amongst these
347 groups. Severe RSV disease and FI-RSV vaccine-enhanced disease are characterized by cellular
348 infiltration and lung hyperinflation (33, 34). We found that lungs from mice immunized with FI-
349 RSV were significantly larger and were > 30% heavier than lungs from the placebo controls,
350 while lungs from VLP vaccinated mice did not differ from the placebo group (Fig. 6 C).

351

352 **DISCUSSION**

353 Here we present data on the immunogenicity, efficacy and safety of a novel VLP based
354 RSV vaccine constructed with different conformations of the RSV F glycoprotein. Previously
355 tested subunit vaccines were formulated primarily with RSV F in its postfusion conformation
356 (35, 36). Recent studies (21, 22, 37) however, have demonstrated that the RSV F in prefusion
357 conformation has the ability to elicit higher levels of neutralizing antibodies than the postfusion
358 conformation. Indeed, different groups have shown that vaccines containing RSV F in the
359 prefusion conformation were superior in protecting mice and cotton rats from RSV infection (21,
360 22, 37). Considering these data, we designed, produced and tested a novel recombinant stabilized
361 prefusion F that is highly expressed in mammalian cells, is incorporated into VLPs, and is
362 recognized strongly by the 5C4 mAb, which binds the prefusion-only antigenic site ϕ .

363 Although VLPs are strong immunogens, we included an adjuvant in the VLP vaccine in
364 order to elicit the greatest immunogenicity. Protection against RSV may require a greater
365 immunity than that stimulated by natural infection, which does not prevent reinfection. We
366 selected the squalene-based oil emulsion because it is a potent inducer of both Th1- and Th2-
367 mediated immunity, is well tolerated and safe (38, 39).

368 Assessment of the protective efficacy afforded by VLP vaccination after RSV virus
369 challenge showed that each one of the three VLP vaccine formulations protected the lungs from
370 viral infection. Furthermore, evaluation of serum neutralization potency showed that the
371 prefusion F VLP vaccine induced antibodies with higher neutralization power than did
372 immunization with the postfusion F VLP vaccine in agreement with previous studies (21, 22),
373 although not to the same extent. However, the VLP combo vaccine showed the best
374 neutralization activity reaching a neutralizing power that was >4 fold greater than that seen with

375 the postfusion VLP vaccine, and >2 fold greater than that seen with the prefusion VLP vaccine.
376 Notably, all VLP vaccines contained the same total F protein content (4 µg total), suggesting that
377 the combination of the two conformations of F protein may be synergistic in eliciting a
378 protective immune response. Recent studies performed with the Newcastle disease virus VLP
379 vaccines showed similar results when comparing postfusion and prefusion forms, however a
380 combo formulation was not tested (21, 40). It seems reasonable to speculate that the combo
381 vaccine displays a larger repertoire of neutralizing epitopes than either of its components. This
382 outcome is significant for RSV vaccine development and requires further investigation to define
383 the underlying mechanism.

384 Consistent with the neutralization assay data, the IgG isotyping analysis showed that the
385 combo vaccine induced a balanced Th1-mediated immune response. On the other hand, the FI-
386 RSV vaccine elicited high levels IgG that did not correlate with the induction of neutralizing
387 antibodies. This outcome clearly illustrates the dichotomy between high antibody titers and
388 neutralizing capacity, which has been the hallmark of the FI-RSV vaccine enhanced disease.

389 Cytokine analysis showed that all VLP vaccinated mice produced statistically significant
390 levels of IFN γ as compared to placebo, which is a correlate of induction of a Th1 type of immune
391 response. On the other hand, the FI-RSV vaccine induced an extraordinary level of IFN γ , and
392 TNF α , as well as a high production of Th2 polarizing cytokines (IL-4, IL-5 and IL-13), Th17
393 polarizing cytokines (IL-17, IL-1 β) and IL-10, and chemokines (Eotaxin, MCP-1, MIP-1 α , and
394 RANTES) all associated with enhanced RSV pathogenesis (24–29). In contrast, VLP vaccinated
395 mice expressed much lower amounts of these inflammatory cytokines and chemokines.
396 Furthermore, histopathology studies showed that VLP vaccination did not induce the detrimental

397 immune cell infiltration inside the lung and that the VLP combo formulation was the best
398 tolerated vaccine.

399 In summary, we describe the production of RSV VLPs composed of RSV F glycoprotein
400 that display different epitopes suitable for the elicitation of neutralizing antibodies. Considering
401 the diversity, neutralizing strength and distribution of epitopes both shared and unique, between
402 the two conformations of F, it seemed important to compare the immunogenicity and efficacy of
403 single VLP vaccines (postfusion or prefusion F) with a combo formulation. This study showed
404 that the VLP combo vaccine, comprised of the multiple epitopes revealed in the postfusion and
405 prefusion F, afforded complete protection against RSV and elicited production of the highest
406 level of serum neutralizing antibodies that correlate with the development of a strong Th1-
407 immune response. Furthermore, immunization with this vaccine proved to be safe, a condition
408 that must be satisfied by any RSV vaccine candidate. We anticipate that the VLP combo vaccine
409 may elicit a broader spectrum of neutralizing antibodies and thus afford better protection against
410 RSV and is a viable safe and efficacious candidate for clinical development.

411

412 **ETHIC STATEMENT**

413 The Institutional Animal Care and Use Committee (IACAC) of the New York Medical
414 College approved the protocol for the mice study. Study were performed in compliance with the
415 approved IACUC protocol (#33-2-0513H) and the Institutional Biosafety Committee policies,
416 and performed under strict accordance to the Office of Laboratory Animal Welfare (OLAW)
417 guidelines, and the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory
418 Animals (NIH).

419

420 COMPETING INTEREST

421 VC, HB, JMG are employees of TechnoVax Inc., BB is employee of EMD-Millipore
422 Corporation. I have read the journal's policy and the authors of this manuscript have the
423 following competing interests: VC, HB and JMG are the inventors on patent related to this
424 publication. This does not alter our adherence to all Clinical and Vaccine Immunology journal
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426

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573 **FIGURE LEGENDS**

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575 **Figure 1) Development of RSV F constructs using structural vaccinology.**

576 **A)** Schematic representation of wild type (WT) RSV F primary structure. F protein
577 matures by furin enzyme cleavage at sites I and II generating F2-F1 protomer and releasing p27
578 glycopeptide. F protein is characterized by heptad repeat domains HRA, HRB and HRC, fusion
579 peptide (FP), transmembrane domain (TM), and cytosolic tail (CT), which is important for virion
580 assembly with the matrix M protein. F elicits neutralizing antibodies able to recognize the
581 antigenic sites: ϕ , I, II, and IV. The figure includes: schematic picture of postfusion hybrid
582 construct (Post) with swapped CT with the analogous domain of the hMPV F (green color);
583 schematic representation of prefusion hybrid construct (Pre) with disulfide bonds modification
584 102-148 and 155-290. **B)** Tridimensional structure representation of F protomer in postfusion
585 and prefusion conformation. The cysteine modifications A102C and I148C are indicated in red.
586 Prefusion conformation is maintained by formation of cysteine link between F2 (purple) and F1
587 (blue) chains (the positions of amino acids 102 and 148 are approximated in the postfusion
588 structure representation). **C)** Dot blot analysis showing the immune reactivity of recombinant
589 RSV F proteins. The graph represents the results of 3 independent experiments for 5C4
590 normalized over palivizumab immune-reactivity: the asterisk indicates a statistically significant
591 p-value ($p<0.05$) between the postfusion and prefusion conditions; ND stands for not detected.
592 Included below is a figure of a single dot blot experiment.

593

594

595 **Figure 2) Structure and Morphology of RSV VLPs using recombinant RSV F and hMPV**

596 **M.**

597 **A)** Drawing of RSV viral particle: F, G, SH, M, M2, P, N, and L proteins, and genomic
598 negative-sense RNA are indicated. **B)** Western blot analysis of RSV VLPs using different
599 antibodies such as goat Anti-RSV (Anti-RSV Gt), Palivizumab, and anti-hMPV M. The Western
600 blotting experiment includes purified RSV virus control. **C)** Purified RSV VLPs were negatively
601 stained and examined by electron microscopy. The micrograph shows spherical and irregular
602 particles (90-100 nm) decorated with surface projections or “spikes” (arrowheads) resembling
603 the morphology of RSV F. **D)** Electron micrographs of immunogold-labeled RSV VLPs probed
604 with the humanized monoclonal antibody Palivizumab and developed with a goat anti-human
605 antibody coupled to gold spheres (10 nm). Detection of gold spheres demonstrates that F is
606 decorating the surface of the VLPs.

607

608 **Figure 3) VLPs vaccine protects against RSV infection.**

609 **A)** Plaque assay analysis of viral titers in the mouse lungs 4 days post-challenge shows
610 undetectable viral replication in VLP vaccinated mice, whereas the placebo control group
611 demonstrates a very productive infection; dotted line indicates the lower detection limit. **B)** Viral
612 micro-neutralization assay shows level of serum neutralizing antibody after vaccination, VLP
613 combo vaccination resulted in a statistically significant enhancement of neutralizing antibody
614 with respect to VLP postfusion and prefusion vaccination ($p<0.05$)*. **C)** Measurement of RSV-
615 specific IgG1 and IgG2a serum antibody titers. Sera collected at day 28 post-immunization (n= 4
616 per group) were assayed for RSV-specific IgG isotypes by ELISA. Results are presented as the
617 reciprocal of the log₂ serum endpoint dilution. Shown antibody titers in the vaccinated mice are

618 statistically significant with respect to the placebo control ($p<0.05$)*. **D)** Analysis of the ratio
619 between IgG2a and IgG1 demonstrates that VLP combo vaccine induces a superior Th1-
620 mediated response. The results in Figure 3 were generated using 4 mice per each condition.

621

622 **Figure 4) Cytokines response in VLPs vaccinated mice lung after RSV infection.**

623 **A)** Cytokines for Th1-mediated response are: IFN γ , IL-12p40, and TNF α . **B)** Th2-
624 mediated response cytokine measurement includes IL-4, IL-5, and IL-13. **C)** IL-17 and IL1- β
625 analysis indicates Th17-mediated response. **D)** IL-10 cytokine demonstrates immune-regulatory
626 process development. Results were generated using a group of 4 mice per each condition 4 days
627 post-infection. The asterisk indicates statistically significant differences between the
628 experimental condition and the placebo control; differences between the FI-RSV and VLP
629 vaccines were statistically significant ($p<0.05$) in all groups, with the exception the prefusion in
630 the IFN γ assay; “ND” indicates not detectable.

631

632 **Figure 5) Chemokine responses in VLP vaccinated mouse lung after RSV infection.**

633 Analysis includes Eotaxin, MCP-1, MIP-1 α , and RANTES. Results were generated
634 using a group of 4 mice per each condition 4 days post-infection; the asterisk indicates a
635 statistically significant difference between an experimental condition and the placebo control
636 ($p<0.05$). Differences between the FI-RSV and the VLP vaccines are also statistically significant
637 ($p<0.05$) in all groups.

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641 **Figure 6) RSV VLP vaccines do not induce “vaccine-enhanced disease” in murine lung.**

642 **A)** Hematoxylin and eosin staining of mouse lung 4 days after viral challenge:
643 microscopic examination of lungs from combo vaccination shows the absence or minor
644 pulmonary pathology. On the other hand, the FI-RSV vaccination induces a very high
645 perivascular immune infiltration. A placebo control is included as a reference. **B)** Perivascular
646 infiltration was scored by blind evaluation of hematoxylin and eosin stained sections of mouse
647 lung 4 days after viral challenge, the score ranges from 0 for normal to maximum of 3 for
648 massive infiltration, the asterisk indicates a statistically significant difference between VLP
649 vaccination versus FI-RSV ($p<0.05$). **C)** Mouse lung was harvested 4 and 7 days post RSV-
650 challenge and weighed; the asterisk indicates statistically significant difference with respect to
651 the VLP vaccines and placebo control ($p<0.05$).











