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Antigenicity and immunogenicity of unique prefusion-mimic F proteins presented on enveloped virus-like particles

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ABSTRACT

Pre-fusion stabilizing mutations (DS-Cav1) in soluble fusion (F) proteins of human respiratory syncytial virus (RSV) were previously reported. Here we investigated the antigenic and immunogenic properties of pre-fusion like RSV F proteins on enveloped virus-like particles (VLP). Additional mutations were introduced to DS-Cav1 (F-dcmTM VLP); fusion peptide deletion and cleavage mutation site 1 (F1d-dcmTM VLP) or both sites (F12d-dcmTM VLP). F1d-dcmTM VLP and F12d-dcmTM VLP displayed higher reactivity against pre-fusion specific site Ø and antigenic site I and II specific monoclonal antibodies, compared to F-dcmTM VLP with DS-Cav1 only. Mice immunized with F1d-dcmTM VLP and F12d-dcmTM VLP induced higher levels of DS-Cav1 pre-fusion specific IgG antibodies, RSV neutralizing activity titers, and effective lung viral clearance after challenge. These results suggest that cleavage site mutations and fusion peptide deletion in addition to DS-Cav1 mutations have contributed to structural stabilization of pre-fusion like F conformation on enveloped VLP, capable of inducing high levels of pre-fusion F specific and RSV neutralizing antibodies.

1. Introduction

Human respiratory syncytial virus (RSV) is a frequent cause of severe bronchiolitis and pneumonia in the lower respiratory tracts in infants and young children as well as in the elderly, resulting in over 3.5 million requiring hospitalization, and 66,000 to 199,000 deaths [1]. Since licensed vaccines are not available, effective vaccines are an urgent need.

Palivizumab is a licensed drug of RSV neutralizing monoclonal antibody (mAb) against fusion (F) glycoprotein and prophylactically used in high risk children [2]. A positive correlation was reported between high titers of neutralizing antibodies and protection of children [3] and the elderly [4] against natural RSV infections as well as protection in human volunteers after RSV challenge [5]. These studies indicate important roles of neutralizing antibodies in providing protection against RSV. Conversely, a recent clinical study demonstrated that antibody concentrations to RSV F have correlations with decreasing RSV disease severity in newborn and infant ages [6]. Therefore, correlates of protection against RSV disease appear to be complex and might be different between passive and active immunity.

The RSV F glycoprotein mediates fusion during virus entry into the host cell membrane and is an important target for inducing neutralizing antibodies to confer protection against RSV [7]. RSV F is synthesized as an inactive precursor that is post-translationally cleaved by furin protease at two polybasic sites, releasing 27 amino acids (P27) and then becomes fusion competent [8]. Furin cleavage generates F1 and F2 subunits covalently linked by two disulfide bridges, forming a metastable conformation, called the prefusion (pre-F), that is membrane-anchored and incorporated onto the surface of budding virions [7–10]. There is an alternative view suggesting that the second furin site cleavage in pre-F occurs in the fluid-filled macropinosomes after endocytosis for fusion into the target cells [11].

A significant portion of neutralizing antibodies in human individuals and IgG preparations was directed against the epitopes present in the pre-fusion (pre-F) form of RSV F [12,13]. This study suggests that a prefusogenic conformation with P27 linkage before the second cleavage might be a transitional conformation during RSV fusion entry and infection into the target cells. Soluble pre-F

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stabilized-forms containing mutations (named DS-Cav1) and a foldon trimer-stabilizing domain could induce higher levels of neutralizing antibodies than soluble post-fusion (post-F) F proteins in animals [10,14].

Therefore, pre-F conformation F represents a promising immunogen for a subunit RSV vaccine. Nonetheless, it remains unknown whether soluble pre-F stabilized proteins with foldon trimers would mimic pre-F conformation F with transmembrane (TM) domains naturally occurring on enveloped RSV. Co-expression of viral structural proteins and surface glycoproteins in mammalian or insect cells produces enveloped virus-like particles (VLP) incorporating viral proteins, mimicking an enveloped virus such as RSV. Newcastle disease virus (NDV) structural proteins (NP, M) were co-expressed in avian cells to produce VLP vaccines containing the ectodomains of RSV glycoproteins [15,16]. Our previous studies demonstrated the immunogenicity and efficacy of RSV F VLP produced in insect cells by co-expressing influenza virus M1 protein [17,18]. RSV F containing VLP vaccines were shown to confer protection against RSV without causing lung inflammation after RSV challenge [15,16,19] whereas soluble RSV F protein vaccination primed immune responses causing lung inflammation after RSV challenge in mice [20]. A recent study by Cullen et al. (2019) reported that higher RSV neutralizing antibodies were induced in cotton rats by NDV VLPs containing pre-F stabilized single chain F with deletions of the P27 sequence and cleavage sites and point mutations, compared to NDV VLP with DS-Cav1 F, despite similar site Ø antigenic properties [21].

Previous studies by Smith et al. and Raghunandan et al. reported the near full-length RSV prefusogenic F nanoparticle vaccine with P27 linkage, inducing palivizumab (site II) competing antibodies and conferring protection in cotton rats after active and passive immunization [22,23]. In a follow up study, prefusogenic F nanoparticle vaccine with P27 linkage was shown to induce neutralizing antibodies competitive with monoclonal antibodies targeting to other antigenic sites present on pre-F and post-F conformation [24]. RSV F mutant proteins containing mutations of one furin site cleavage and fusion peptide (FP) deletion were demonstrated to enhance the expression of F proteins on insect cell surfaces [22].

RSV F protein furin site cleavage was reported to induce structural changes in F proteins (from cone- to lollipop-shapes; pre-F to post-F) and aggregation in anchorless soluble F proteins [8,25,26]. Full-length soluble F protein showed no binding reactivity for site Ø epitope specific mAb of 5C4, suggesting post-F conformation [27]. However, soluble post-F protein with FP deletion or uncleaved soluble post-F protein displayed low levels of binding reactivity for site Ø epitope specific mAbs, 5C4 and D25 compared to DS-Cav1 pre-F proteins [14]. P27 linkage in uncleaved soluble F protein with a trimer stabilizing domain (GCNt) was reported to enhance binding reactivity for pre-F site Ø specific mAbs, AM22 and D25 [28]. In contrast, FP deletion in post-F/F with foldon in NDV VLP could not show significant binding reactivity for site Ø epitope specific 5C4 mAb [27], suggesting a difference in prefusion stabilizing mutations required in soluble protein and with TM anchoring on enveloped particles.

These previous studies suggest that furin cleavage and FP as well as DS-Cav1 mutations affect expression and conformational neutralizing epitope exposure of RSV F proteins, differentially depending on soluble proteins or F proteins with TM anchored on enveloped VLP. The antigenic and immunogenic properties of these RSV F mutant proteins in enveloped VLPs largely remain unknown. In this study, we generated unique pre-F stabilized combination mutants containing DS-Cav1 and other additional mutations in the furin cleavage sites and FP deletion and presented these F mutants on membrane-anchored VLPs. The antigenic and immunogenic properties of pre-F and post-F conformation F proteins anchored on enveloped VLPs were further investigated. Unique pre-F stabilized VLP constructs containing combination mutations of DS-Cav1, furin cleavage sites, and fusion peptide deletion were found to be highly effective in inducing antibodies specific for pre-F antigens and protection.

2. Materials and methods

2.1. Cells, virus, and reagents

Spodoptera frugiperda 9 (SF9) insect cells (CRL-1711; ATCC) were maintained in suspension in serum-free SF900-II medium (GIBCO-BRL) and used for production of recombinant baculoviruses (rBVs) and VLPs. RSV A2 and A2-K-line19F were used as described [19]. Monoclonal antibodies (mAb) D25 and 131-2A were purchased from Creative Biolobs and from Millipore. Pre-F protein with DS-Cav1 mutations and post-F protein, and 5C4 mAb were generously provided from Dr. Graham (VRC, NIAID, NIH). Palivizumab mAb was kindly provided by Dr. Eun-Hyung Lee (Emory University). HRP conjugated anti-mouse antibody IgG, IgG1, IgG2a, and anti-human antibody IgG were purchased from Southern Biotech (Birmingham, AL).

2.2. Preparation of RSV F mutant VLP constructs

A full-length human codon-optimized RSV A2 F DNA was previously described [29]. To introduce DS-Cav-1 mutations into F protein, four primers were used for over-lap PCR as follow: S155C (5′ AAAGCTAGCGGAG TGGCCGTGTG TAAGGTGC), S290C (5′ GATGATGCAC ATGATGGAGT AGCTCTGCTG), S190F (5′ GCTTGTCGAT GTAGTTCTTC AGATCCAGCA CCTTGAAGGTCAG), and V207L(5′ CTGAAGAACT ACATCGACAA GCAGCTGCTG CCCATCCTGAACAAG). The PCR products of full-length F gene including DS-Cav-1 mutations were cloned in pFastBac1 plasmid to construct F-dcmTM (DS-Cav1). Mutation of furin site 1 KKRKRR136 to KQKQQ136 and amino acid residues 137–147 were reported to increase the expression of F proteins on the insect cell surfaces [22]. Various mutations (RANN, NARR, NANR, KARK, KAKK) in the furin site RARR109 were previously reported [8,28,30]. Additional mutations including deletion of 8 amino acid residues (aa137-144) in fusion peptide region and in the furin cleavage site 1 (F1d-dcmTM VLP) or both cleavage 1 and 2 sites (F12d-dcmTM VLP) are depicted in the Fig. 1, using the following primers: F1m (Forward; 5′ AAA *ACT AGT* GCC ATT GCT AGC GGA GTG GCC GTG TCA AAG GTG CGCT GCC, Reverse; AAA *ACT AGT* CTG CTG CTT CTG CTT CTT GCT CAG GGT CAC GTT GG), F2m (Forward; 5′G ATG CAG AGC ACC CCC GCC ACC AAC AAC AAG GCT AAA AAA GAG CTG CC, Reverse; 5′GT GTA GTT CAT GAA CCG AGG CAG CTC TTT TTT AGC CTT GTT GGT G). An amino acid change (G → T) by *Spe*I site insertion (italic font codons in F1m forward primer) was depicted in the Fig. 1B. Mutant F and wild type (WT) F with TM on enveloped VLPs were produced in Sf9 insect cells infected with rBVs expressing RSV F and influenza M1 core proteins, and characterized as previously described [17]. Briefly, Sf9 insect cell culture supernatants were collected by centrifugation (6000 rpm, 20 min) to remove insect cells. The cleared supernatants containing F VLPs were purified by ultracentrifugation (30,000 rpm, 1 h). The F protein contents in F mutant VLPs were determined in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% SDS-PAGE) followed by Western blot using D25 mAb and HRP-conjugated anti-human IgG antibody (Southern Biotech).

2.3. Enzyme-linked immunosorbent assay (ELISA)

For characterization of different mutant F protein VLPs (Fig. 1), 96-well microtiter plates were coated with the serial diluted F

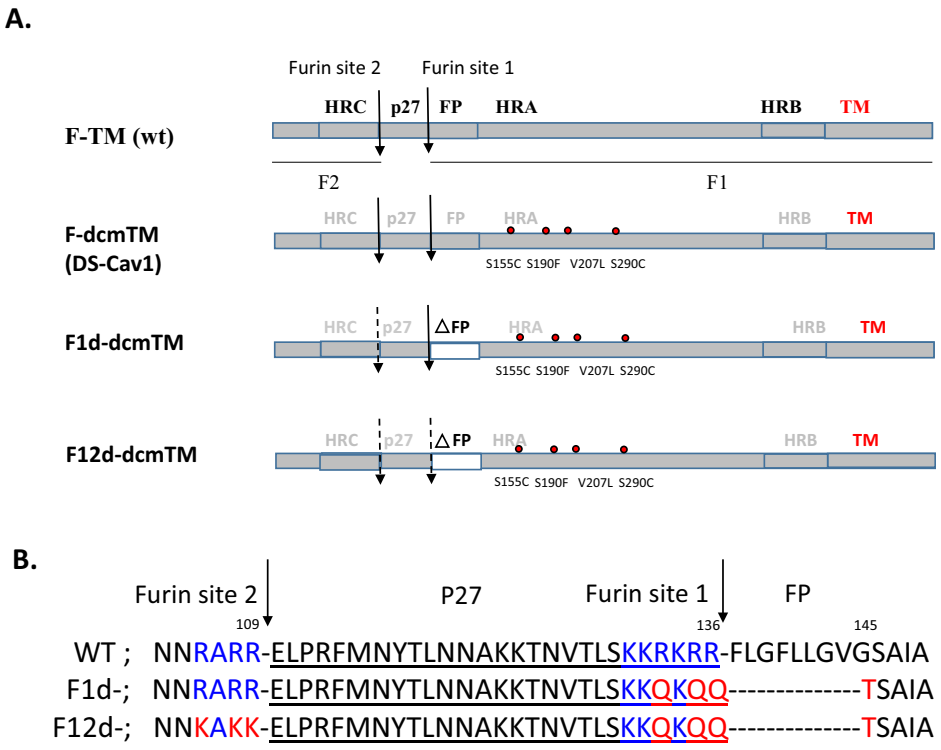

Fig. 1. Diagram of mutant F gene constructs to be presented on VLPs (A) The modified full-length RSV F genes in VLP, furin protease cleavages (furin 2, furin 1, black arrowheads) generate F1 and F2 subunit fragments in wild type (WT) F-TM. F-dcmTM (DS-Cav1): A full-length F contains DS-Cav1 mutations known to stabilize pre-F conformation in soluble proteins with foldon [9], DS-Cav1 mutations of S155C, S190F, V207L, and S290C. F1d-dcmTM: mutation in furin site 2, FP deletion, and DS-Cav1. F12d-dcmTM: mutation in furin sites 1 and 2, FP deletion, and DS-Cav1. (B) The modified amino acid sequences in RSV F furin cleavage sites and fusion domain, RSV F furin cleavage site F1d-dcmTM and F12d-dcmTM constructs have a deletion of the fusion peptide (FP) and furin 1 cleavage mutation (RARR → KAKK, F1d-dcmTM) or both furin 1 and 2 cleavage mutations (KKRKRR → KKQKQQ, F12d-dcmTM). P27, excised peptide; FP, fusion peptide; HRA, -B, and –C heptad repeats A, B, and C are indicated; TM, RSV F protein transmembrane domain including a C-terminal tail.

protein mutant VLPs as described [17,31]. The indicated F protein specific mAb was incubated at 4 °C for 16 h and detected by anti-mouse or anti-human HRP-conjugated IgG. For analysis of mouse sera by an ELISA, formalin inactivated RSV (FI-RSV, 150 ng/50 μL) prepared as described [32], DS-Cav1 protein (10 ng/50 μL), and post-F protein (10 ng/50 μL) were used to coat the plates. The serum antibodies were detected by secondary anti-mouse HRP-conjugated IgG antibodies and TMB substrate.

2.4. Immunization and challenge

BALB/c female mice (6–8 weeks old, Charles River) were intramuscularly primed and boosted with WT or mutant F VLPs (10 μg) in a 3-week interval (n = 5). For RSV challenge, mice were isofluorane-anesthetized and intranasally infected with 1×10^6 plaque-forming units (PFU) of RSV A2 strain. All animal experiments and husbandry involved in the studies were conducted under the guidelines of approved IACUC protocols at the Georgia State University.

2.5. RSV neutralization assays and lung virus loads

RSV-specific neutralizing antibody titers were measured using red fluorescent RSV A2 -line19F in a micro-neutralization assay [33]. Briefly, mouse sera after RSV A2 challenge infection were heat-inactivated at 56 °C for 30 min and serially diluted two-fold in EMEM media. Equal volumes of the diluted sera were mixed with A2-K-line19F virus (400 PFU/well). Serum and virus mixtures were incubated on Vero cells. The fluorescent viral foci were enumerated by using Synergy H1 hybrid Reader (BioTek), 588 λ ex/635 λ em, log 2 in 95% reduction effective concentration (EC95), and imaged by Zeiss fluorescent microscopy. Neutralizing titers were also determined in serially diluted sera mixed with RSV (400 PFU/well) by an immune-plaque staining method [20]. The infected Vero cell plates were fixed with 10% formalin and then viral plaques were visualized by immunostaining with 131-2A mAb and DAB substrate (Invitrogen).

2.6. Lung histology

For histological analysis of lung inflammation, individual lungs were immersed in 10% neutral buffered formalin for 48 hr, transferred to 70% ethanol, embedded in paraffin, sectioned into a thickness of 6 μm and then stained with hematoxylin and eosin (H&E) [34]. Histology pictures were examined and taken by camera-attached microscope as described [20].

2.7. Statistical analysis

Results are expressed as mean ± the standard errors of the mean (SEM). The statistical analyzes were performed by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison test in GraphPad Prism version 5 (GraphPad Software, Inc.).

3. Results

3.1. Preparation of RSV F constructs with furin-cleavage and DS-Cav1 mutations in a transmembrane form on VLP

We reported that VLP presenting F antigens could be a promising delivery vehicle to avoid vaccine-enhanced RSV disease after challenge compared to F protein vaccine [20]. Pre-fusion F stabiliz-

ing mutations known as DS-Cav1 were reported to display pre-F epitopes in a soluble form linked to the trimer-stabilizing foldon [9]. DS-Cav1 mutations introduce disulfide bonds (DC) [S155C, S290C] and cavity filling [S190F, V207L] to stabilize the pre-fusion conformation [9]. The VLP containing F with DS-Cav1 mutations and transmembrane (TM) domain was named F-dcmTM (Fig. 1A).

FP deletion or P27 linkage to F subunit proteins were shown to help the binding of site Ø specific D25 mAb during conformational transitions [14]. Since P27 linkage between F1 and F2 units has been reported to enhance the binding reactivity for pre-F site Ø specific mAbs, AM22 and D25 in uncleaved F protein in F-GCNt trimerized stabilization [28], P27 peptide was retained in the F mutant constructs via cleavage mutations in both sites (F12d-dcmTM VLP) or one furin cleavage site mutation (F1d-dcmTM VLP). Combinatorial mutations of P27 linkage and FP deletion were introduced, expecting additional stabilization of pre-F conformation in the F1d-dcmTM and F12d-dcmTM VLP constructs compared to F-dcmTM VLP of DS-Cav1 mutation only. F1d-dcmTM VLP was generated to contain F with deletion of the first 8 residues (aa136-144) of the FP domain and mutations in the furin cleavage site 1 (KKRKRR → KKQKQQ) adjoining the F1 major domain (Fig. 1B). The F12d-dcmTM VLP was designed to contain additional cleavage mutations in both furin cleavage sites 1 and 2 (Fig. 1B).

3.2. Combination mutations of furin cleavage and DS-Cav1 F proteins in VLP enhance antigenicity against the sites Ø and II specific antibodies

To determine the levels of antigenic display, we measured reactivity of two pre-F site Ø specific mAbs, D25 and 5C4, and site I and site II specific mAbs, 131-2A and palivizumab, respectively (Fig. 2). F1d-dcmTM VLP and F12d-dcmTM VLP displayed the highest levels of reactivity against D25 and 5C4 mAbs recognizing the site Ø in pre-F conformation. F-dcmTM VLP exhibited substantial levels of reactivity against D25, 5C4 mAb, which were higher than F-TM but lower than F1d-dcmTM VLP and F12d-dcmTM VLP (Fig. 2A and B). F1d-dcmTM and F12d-dcmTM VLPs displayed highest reactivity against the site II specific palivizumab, followed by F-dcmTM and then WT F-TM (Fig. 2C). High reactivity against antigenic site I mAb 131-2A was observed with F1d-dcmTM, F12d-dcmTM, and F TM VLPs, but low activity with F-dcmTM VLP (Fig. 2D). These ELISA antigenic data suggest that F1d-dcmTM and F12d-dcmTM VLP constructs display higher reactivity to mAbs recognizing pre-F site Ø epitope as well as sites I and II mAbs, compared to the F-dcmTM containing DS-Cav1 mutation only. Combination mutations of DS-Cav1, FP deletion, and P27 linkage are likely contributing to stabilizing pre-F conformation with high reactivity against sites I and II epitope mAbs. In the western blot data of SDS-PAGE (Supplementary Fig. S1), little less density of F protein band was observed in F-dcmTM sample compared to F1d-dcmTM or F12d-dcmTM samples. It is also possible that displaying more molecules of RSV F protein per VLP particle in the F1d-dcmTM VLP and F12d-dcmTM VLPs has contributed to higher binding to RSV F specific mAbs, compared to F-dcmTM VLP.

We attempted to predict a structure-based model for DS-Cav1 protein with P27 linkage and FP deletion by using SWISS-MODEL protein structure homology-modelling [35]. SWISS-MODEL of F12d-dcmTM predicted that P27 linkage to the F2 and F1 subunit with FP deletion might stabilize the heptad repeat A (HRA) domain in a DS-Cav1 prefusion cone shape (Supplementary Fig. S2). In this modeling, the linkage of P27 in FP deletion between F1-α2 and F2-α1 helix domains enables the interaction with F1-α2 in HRA, likely contributing to stabilization of HRA domains in pre-fusion cone-shape, keeping away from extension to pre-hairpin conformation (Supplementary Fig. S2C). The epitope sites Ø, I, II, III, IV, and P27 peptide could be located on the surfaces of a stabilized cone-shape conformation in the structure model (Supplementary Fig. S2), consistent with the antigenic properties of F12d-dcmTM VLP (Fig. 2).

3.3. F1d-dcmTM and F12d-dcmTM VLPs effectively induce antibodies reactive to a pre-F conformation antigen

A profile of antibodies induced by vaccination with F-dcmTM, F1d-dcmTM and F12d-dcmTM was determined by ELISA using FI-RSV and post-F protein antigens or DS-Cav1 pre-F protein antigen. The subsets of antigen-specific T helper (Th) cells regulate the differentiation and activation of B cells secreting IgG isotype antibodies [36,37]. Determination of IgG isotypes indicates the underlying Th1 or Th2 response in mice [36,37]. Th1 (interferon-γ) cytokine promotes the production of IgG2a isotype and Th2 (interleukin-4) cytokine IgG1 isotype antibodies [36,37]. The mice that were intramuscularly immunized with WT F-TM VLP induced lower levels of IgG, IgG1 (Th2), and IgG2a (Th1) isotype antibodies binding to DS-Cav1 pre-F antigen but higher levels of IgG to FI-RSV and post-F, compared to those by F1d-dcmTM or F12d-dcmTM VLP vaccination as determined by ELISA (Fig. 3A–C). F-dcmTM VLP immunized mice induced Th1 type IgG2a dominant isotype antibodies binding to FI-RSV and post-F rather than DS-Cav1 pre-F antigens as shown by higher IgG2a/IgG1 ratio data (Fig. 3B and C). Thus, F-dcmTM VLP appears to be immunogenic in inducing IgG antibodies recognizing FI-RSV and post-F antigens despite lower antigenic reactivity to pre-F site Ø, site I and site II epitope recognizing mAbs. Mice with WT F-TM VLP or F-dcmTM VLP vaccination did not induce significant levels of serum antibodies binding to DS-Cav1 pre-F protein antigens (Fig. 3A). F1d-dcmTM or F12d-dcmTM VLP vaccination of mice induced higher levels of IgG, Th1 type IgG2a dominant isotype, and less Th2 type IgG1 antibodies specifically binding to DS-Cav1 pre-F protein antigens as indicated by high IgG2a/IgG1 ratios (Fig. 3A), but low levels of antibodies specific for FI-RSV and post-F with FP deletion antigens (Fig. 3B and C). These results suggest that mice immunized with F1d-dcmTM and F12d-dcmTM VLP constructs with combination mutations of DS-Cav1, furin cleavage site, and FP deletion are effective in inducing Th1 type IgG2a dominant antibodies reactive to DS-Cav1 pre-F protein antigens. In contrast, F-dcmTM VLP was not effective in inducing IgG antibodies specific for DS-Cav1 pre-F protein antigens.

To further support the conformation dependent recognizing IgG antibody responses, we determined the antigenic properties of RSV F proteins used in ELISA coating antigens (Supplementary Fig. S3). Foldon-stabilized DS-Cav1 pre-F protein antigen [9] displayed higher levels of reactivity against D25 and 5C4 pre-F specific mAbs than post-F protein antigens with FP (aa residues 137–146) deletion [38] (Supplementary Fig. S3), which is consistent with another study on post-F antigen with FP deletion [14]. Post-F protein antigens with FP deletion showed higher reactivity against site I specific mAb, 131-2A than DS-Cav1 pre-F antigen (Supplementary Fig. S3). Both pre-F and post-F protein antigens exhibited a similar profile of site II specific palivizumab reactivity.

3.4. Higher RSV neutralizing titers and effective lung viral clearance are induced by F1d-dcmTM and F12d-dcmTM VLP vaccination

Mouse sera collected at week 3 after boost immunization were heat-inactivated and used to determine the capacity to neutralize RSV infection in Vero cells using RSV A2 or RSV A2-K-line 19F (Fig. 4A, Supplementary Fig. S4A and B). The different RSV neutralizing capacities were observed in the red fluorescent images of the RSV A2-K-line 19F infected Vero cells (Supplementary Fig. S4A). WT F-TM or F-dcmTM VLP immune sera could not neutralize 95%

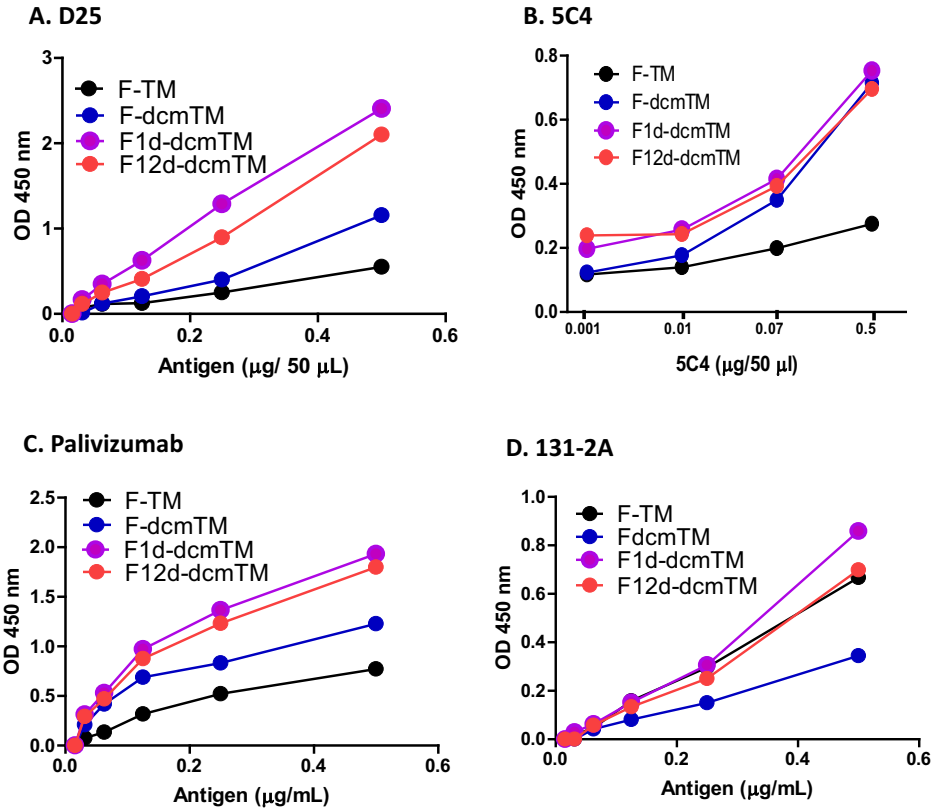

Fig. 2. Antigenic profiles of mutant RSV F proteins in TM VLPs WT (F-TM) and mutant F protein (F-dcmTM, F1d-dcmTM, F12d-dcmTM) VLPs were coated in serial dilution on 96-well plates. (A) 1 μg/mL of D25 mAb specific for pre-F site Ø epitope. (B) 5C4 mAb specific for pre-F site Ø epitope, F mutant VLPs (0.25 μg/50 μL) were coated on 96-well plates, then 5C4 mAb in serial dilutions were used as indicated. (C) 1 μg/mL of Palivizumab specific for the F site II epitope. (D) 1 μg/mL of 131-2A mAb specific for site I epitope.

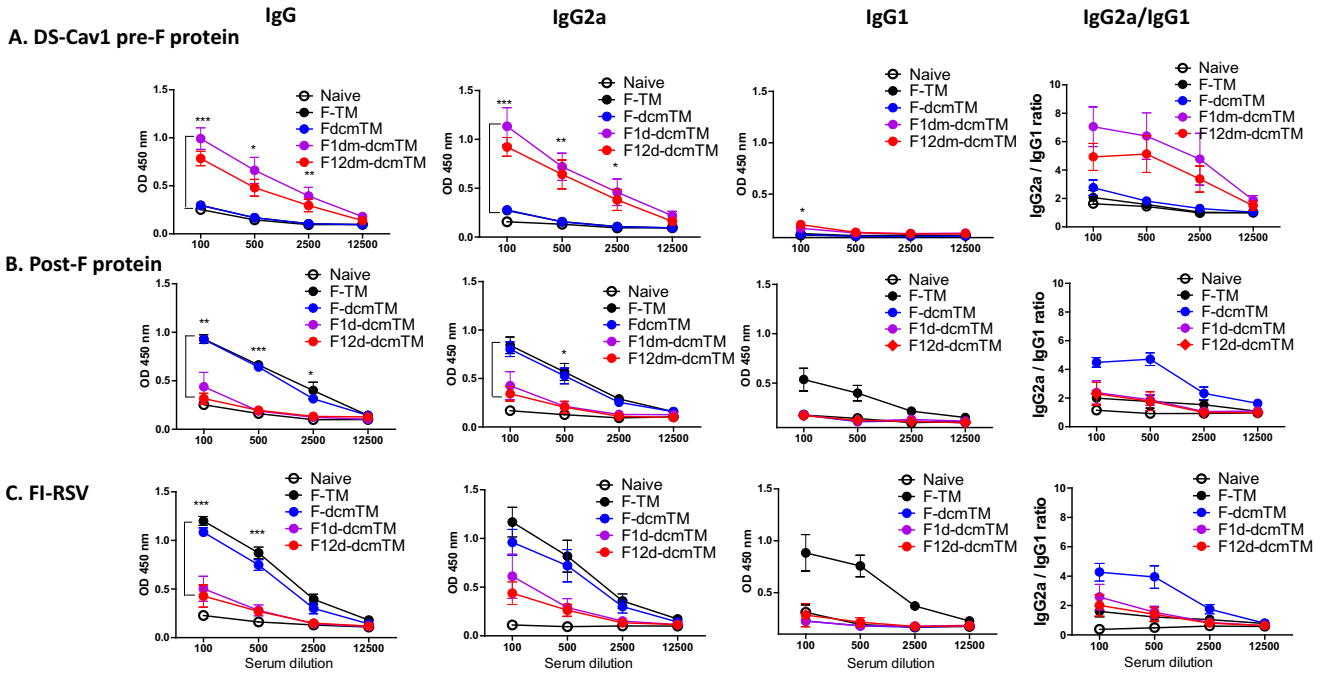

Fig. 3. F1d-dcmTM and F12d-dcmTM VLP immunized mice preferentially induce Th1 type IgG2a isotype antibodies specific for pre-F protein antigens Levels of serum IgG isotype antibodies and IgG2a/IgG1 ratio specific for FI-RSV, post-F protein, and pre-F (DS-Cav1) protein antigens were determined by ELISA in the groups of mice (n = 5) at 3 weeks after boost. (A) IgG and isotypes specific for FI-RSV. (B) Post-F protein specific IgG and isotypes. (C) Pre-F (DS-Cav1) protein specific IgG and isotypes; Naïve: Unimmunized mice, F-TM: WT F-TM VLP, F-dcmTM: F-dcmTM VLP, F1d-dcmTM: F1d-dcmTM VLP, F12d-dcmTM: F12d-dcmTM VLP. Results are presented as means ± SEM and statistical analysis between F-dcmTM VLP and F1d-dcmTM VLP or F12d-dcmTM VLP was performed by one-way ANOVA with Tukey's multiple comparison test in GraphPad Prism; *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$.

even in lowest serum dilutions. In consistent, the higher neutralizing titers with sera of mice immunized with F1d-dcmTM VLP or F12d-dcmTM VLP were detected by an immunostaining plaque assay in inhibiting the infection of RSV A2-K-line 19F or RSV A2 than those of WT F-TM or F-dcmTM VLP (Fig. 4A, Supplementary Fig. S4B and C). The sera of mice immunized with F1d-dcmTM VLP or F12d-dcmTM VLP were observed to exhibit higher neutralizing titers (serum dilution of neutralizing 95%, 12–13 log2) of inhibiting the infection of fluorescent RSV A2-K-line 19F compared to that of WT F-TM or F-dcmTM VLP (5 in log2) in the high dose of virus inoculation (400 PFU/well of Vero cells) (Fig. 4A).

At day 5 after challenge with RSV A2 (1×10^6 PFU), lung viral loads were determined (Fig. 4B). The unimmunized control mice showed the highest levels of lung RSV titers. Also, the WT F-TM and F-dcmTM VLP groups were able to reduce lung viral loads by 20 – 50 folds compared to those in naïve mice with RSV infection but still showed substantial levels of lung RSV titers. Lung viral loads were below the limit of detection (LOD) in the groups of mice with F1d-dcmTM VLP and F12d-dcmTM VLP vaccination (Fig. 4B). These results suggest that F1d-dcmTM and F12d-dcmTM VLP vaccines are effective in inducing RSV neutralizing antibodies and clearing lung viral loads after RSV challenge in mice.

3.5. F1d-dcmTM and F12d-dcmTM VLP does not prime lung histopathology after challenge

We carried out histology of lung tissues collected from mice after RSV challenge. The naïve mice with RSV infection showed a moderate to high level of histopathology, displaying infiltrates around the airways and interstitial spaces (Fig. 5A). All vaccinated mouse groups displayed lower levels of lung histopathology after RSV challenge, compared to naïve mice with RSV infection, indicating no vaccine-enhanced pulmonary inflammation (Fig. 5C–F). The F-TM and F-dcmTM groups (Fig. 5C–D) showed a moderate level of lung inflammation around the airway epithelial lines, correlating with lung viral loads (Fig. 4), compared to the F1d-dcmTM and in the F12d-dcmTM groups (Fig. 5E–F).

4. Discussion

An effective RSV vaccine should overcome two main challenges. One challenge is to induce a protective immune response. Another challenge is to avoid priming vaccine-enhanced respiratory disease after RSV infection [39]. A previous study reported that low doses of pre-fusion F proteins primed for alveolitis in the cotton rat model independently of a Th1 or Th2 immune modulating adjuvant [40]. Presentation of RSV F proteins on VLP was shown to be effective in priming immune responses and preventing enhanced alveolitis after RSV infection [15,20] whereas vaccination with RSV F soluble proteins was reported to be likely to prime alveolitis upon RSV challenge in animal models [20,41,42]. It is also possible that pre-F and post-F VLP construct vaccines might have induced different immune profiles affecting lung inflammation after RSV infection. In this study, vaccinated mouse groups did not display enhanced pulmonary inflammation after challenge. Therefore, VLP would be a desirable platform to present RSV F protein antigens.

Relative surface area between pre-fusion and post-fusion conformation F proteins were estimated to be the same ratio of 50%, indicating 50% of shared common surface epitopes [13,43]. Nonetheless, an estimate of 70% antibodies in human sera binds to pre-F–specific surfaces, 25% bind to the shared surfaces, and 5% bind to the post-F–specific surfaces, which is disproportionate to the available surface area [13,44]. Previous studies demonstrated that, compared to soluble post-F conformation proteins with or without FP deletion, RSV pre-F soluble proteins containing DS-Cav1 mutations and a trimer stabilizing foldon were more effective in inducing IgG antibodies recognizing pre-F antigens and neutralizing antibodies in animal models [9,14,45]. Therefore, it was reasoned that recombinant RSV F protein constructs with mimicry of pre-F protein conformation would be a promising strategy to develop an effective RSV vaccine candidate.

Additional or alternative mutations in F were previously reported to increase the stability of soluble pre-F proteins. More stable DS-Cav1 mimicking pre-F soluble protein constructs were demonstrated, by replacing P27 and FP with a linker to generate an uncleaved single-chain (SC) F plus triple mutations (N67I, S215P, E487Q) in the refolding regions stabilized with a fibritin domain [45] or F mutant with extended-deletion of 48 residues including P27 plus DS-Cav1 mutation and additional disulfide bonds (D486C, D489C) [46]. A previous study reported the effects of furin cleavage-driven transition on antigenic properties in F proteins by comparing uncleaved DS-Cav1 pre-F protein retaining P27 with two furin site mutations and cleaved DS-Cav1 proteins at furin sites [14]. Soluble post-F protein with FP deletion or uncleaved soluble post-F protein containing furin site cleavage

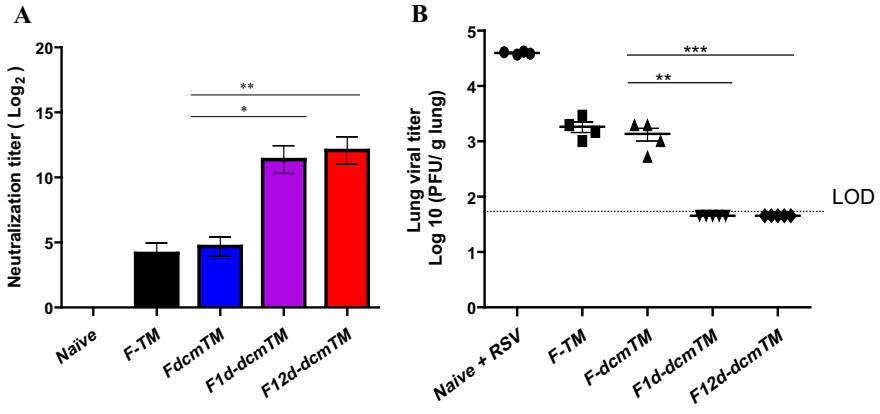

Fig. 4. F1d-dcmTM and F12d-dcmTM VLP immunized mice exhibited high levels of RSV neutralization titers and clear lung viral loads after RSV challenge (A) Serially diluted mouse sera collected at 3 weeks after boost were heat-inactivated and used to determine RSV neutralization activity in Vero cells (approximately 400 PFU per well). Serum neutralization activity against RSV A2-K-line 19F in Vero cells was tittered for 95% reduction in fluorescent signals (λex 588/λem 635). (B) Viral titers in the lungs from individual mice in the groups (n = 5) were determined at day 5 after challenge. Dotted line: limit of detection. Naïve: Unimmunized mice, F-TM: WT F-TM VLP, F-dcmTM: F-dcmTM VLP, F1d-dcmTM: F1d-dcmTM VLP, F12d-dcmTM: F12d-dcmTM VLP. Results are presented as means ± SEM and statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test in GraphPad Prism; ** $p < 0.005$, * $p < 0.05$.

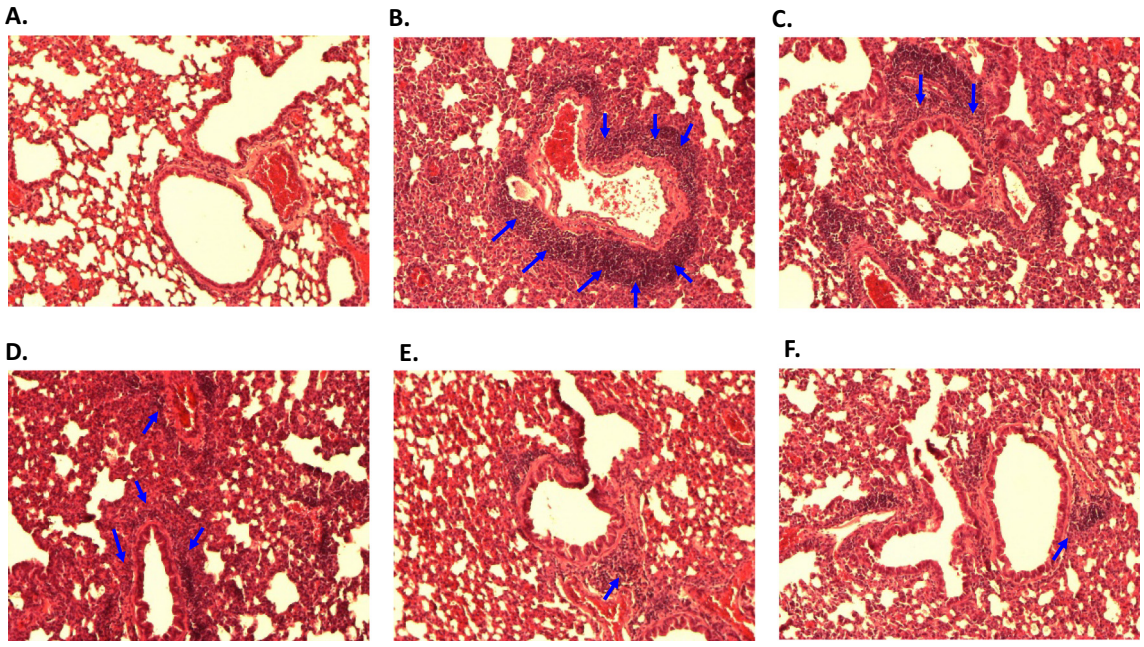

Fig. 5. Pulmonary histopathology in H&E stain at day5 post RSV challenge. Lung tissues were collected from individual mice (n = 5) at day 5 post RSV challenge (5×10^5 PFU/mouse). H&E staining shows a degree of pneumonia in the airways, blood vessels, and interstitial spaces. The arrows (blue) indicate the major anatomical markers on each slide, where the immune pathology spots were observed in the different groups. (A) Naïve, (B) Naïve + RSV, (C) FTM + RSV, (D) F-dcmTM + RSV, (E) F1d-dcmTM + RSV, (F) F12d-dcmTM + RSV. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mutations showed a low level of binding reactivity for Ø epitope specific mAb, 5C4 and D25 [14], indicating that FP deletion or P27 linkage would help D25 mAb bind to F site Ø epitopes during conformational transitions in RSV fusion (pre-F – pre-hairpin – post-F transition conformation). A single chain P27 linkage between F1 and F2 units was reported to enhance the binding reactivity for pre-F site Ø specific mAbs, AM22 and D25 in uncleaved F protein in F-GCNt trimerized stabilization [28]. Therefore, we hypothesized that combined mutations of retaining P27 linkage and FP deletion would further stabilize pre-F conformation in DS-Cav1 mutants F VLP.

Furin site cleavage and FP region on pre-F surface conformation of parainfluenza virus 5 (PIV5) F protein were suggested to be responsible for FP extrusion promoting pre-hairpin conformational change to fuse with host membranes [47]. A conformational change was reported to occur between uncleaved DS-Cav1 protein with both furin cleavage site mutations and cleaved DS-Cav1 protein at furin sites [14]. SWISS-MODEL protein structure homology-modelling of F12d-dcmTM predicts that P27 might be exposed on the surface of DS-Cav1 protein cone-shape (Supplementary Fig. S2). In this modeling, the linkage of P27 in FP deletion between F1-α2 and F2- α1 helix domains may enable the interaction with F1-α2, likely contributing to stabilization of HRA domain, retaining it in pre-fusion conformation, away from transition into a pre-hairpin conformation (Supplementary Fig. S2).

Previous studies about P27 linkage and FP deletion and the prediction of P27 location in structure homology modeling support that DS-Cav1 protein in F12d-dcmTM or F1d-dcmTM VLP would be more stable in retaining pre-F like conformation than F-dcmTM VLP. DS-Cav1 F-dcmTM VLP displayed higher binding activity to pre-F epitope specific mAbs (D25, 5C4) than WT F-TM VLP, suggesting that F-dcmTM VLP exposes pre-F epitopes. Consistent, McGinnes Cullen et al. [27] reported that DS-Cav1 pre-F (+/− foldon) on VLP with chimeric NDV TM displayed high reactivity to site Ø specific antibodies, and similar binding activity to the antibodies for site II and I epitopes [27]. Notably, we found that F1d-dcmTM VLP and F12d-dcmTM VLP containing additional mutations (furin cleavage sites and FP deletion) resulted in higher binding reactivity to the mAbs specific for the pre-F epitope site Ø (D25, 5C4), and site I, II epitopes compared to F-dcmTM VLP. Consistent with this study, McLellan et al. (2013) also reported that combination (DS-TriC) of DS mutations (S155C, S290C) plus TriC mutations (D486H-E487Q-F488W-D489H) generated the foldon-trimer pre-F conformation displaying high antigenic reactivities against multi epitope sites (Ø, I, II, III, IV) [9], suggesting a further stabilized pre-F conformation with high antigenic reactivities against multi site-specific mAbs.

Immunogenicity data of sera from F-dcmTM VLP vaccinated mice revealed more striking differences in inducing IgG antibodies specific for pre-F or post-F RSV antigens. The F-dcmTM VLP group showed high levels of IgG antibodies binding to post-F and FI-RSV antigens as much as WT F-TM VLP, but low IgG isotype antibodies to pre-F protein antigen. Consistent, a similar trend of IgG antibodies binding to post-F antigens at approximately 10 folds higher than IgG levels binding to pre-F antigens was reported in mice vaccinated with 30 μg of DS-Cav1 F foldon proteins on NDV TM VLP [27]. This and other studies suggest that DS-Cav1 mutant protein in membrane anchored F-dcmTM VLP might be unstable for retaining pre-F transition conformation, in contrast to soluble DS-Cav1 pre-F protein with foldon stabilization.

The soluble DS-Cav1 proteins could not be stable in pre-F trimer conformation without foldon, demonstrating an important role of foldon as a stabilizing domain [48]. RSV pre-F protein trimers were shown to be in a dynamic transition state of being dissociated and associated on viral lipid membrane [49], indicating that pre-F TM on lipid membranes in the absence of additional mutations would be less stable than foldon stabilizing domain in pre-F proteins. Consistent, F trimer stabilizing GCNt domain was reported to be essential for maintaining pre-F conformation, by preventing HRA extension to pre-hairpin conformation of F proteins of paramyxovirus [47,50,51].

It is possible that more factors such as P27 linkage and FP deletion might be responsible for stabilizing pre-F TM conformation anchored on enveloped VLP. DS-Cav1 protein with TM domain

anchoring on lipid envelope VLP might be unstable after cleavages at both furin sites and P27 release, more likely resulting in HRA extension close to pre-hairpin conformation as proposed during fusion with target cell membranes [52]. Alternatively, dynamic fluidity of lipid membrane might make pre-F site Ø epitopes be under the pressure of dissociation into extended pre-hairpin structure. The instability of F-dcmTM VLP might induce post-F like conformation, would make pre-F site Ø and other epitopes less available, consistent with low levels of IgG responses to DS-Cav1 pre-F antigen but high levels of IgG antibodies binding to post-F and FI-RSV antigens. Consistent with this study, recombinant PIV5 expressing DS-Cav1 with TM did not induce higher neutralizing titers than WT F [53]. Meanwhile, a structure homology-modelling of F12d-dcmTM predicts that FP deletion, DS-Cav1 and furin site mutations linking P27 to the F1 large subunit might help in holding HRA in a cone-shape pre-fusion state (Supplementary Fig. S2). Thus, FP deletion and retention of P27 linkage might be contributing to stabilizing pre-F conformation of DS-Cav1 mutant F on enveloped VLP, providing unique properties with high antigenic reactivity toward multi epitope neutralizing sites and pre-F immunogenicity.

Nanoparticle vaccine of prefusogenic F protein with TM containing furin 1 cleavage site aa136 mutation and FP deletion has been reported to induce antibodies targeting multiple antigenic sites Ø, II, IV, and VIII [22–24]. In a phase III study with the elderly (≥60 years), the insect cell-derived recombinant RSV F nanoparticle vaccine inducing palivizumab-competing antibodies [22] did not show significant efficacy against RSV moderate severe lower respiratory disease in older adults, partially due to a lower attack rate [54,55]. Serum neutralization titers of both F1d-dcmTM VLP and F12d-dcmTM VLP with FP deletion were significantly higher than WT F-TM or F-dcmTM VLP. High levels of IgG antibodies recognizing pre-F antigens appear to correlate with high neutralizing titers against RSV A2-K-line 19F as observed in immune sera by F1d-dcmTM VLP and F12d-dcmTM VLP vaccination. Different DS-Cav1 mutant F VLPs might represent different discontinuous pre-F transition status depending on furin site cleavage, FP deletion, and TM stabilization, all of which could have impact on differential antigenic and immunogenic properties. It is important to confirm the findings of these constructs in a cotton rat model. Overall, the findings in this study are expected to provide insight into developing effective and safe RSV vaccine candidates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.09.041.

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