# Effect of Epitope-CpG-DNA-Liposome Complex without Carriers on Vaccination of Respiratory Syncytial Virus Infection 

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#### Abstract

Respiratory syncytial virus (RSV) is a common virus related to disease in the lung epithelium of young children and infants. However, RSV vaccine has not yet been developed. Thus it is difficult to develop a whole-RSV vaccine due to induction of Th2-type hyper-immune responses. To overcome this limitation, we used CpG-DNA encapsulated within liposome complex (Lipoplex(O)) as an adjuvant for the induction of a Th1dominated humoral response in animal experiments. However, vaccination with a complex of UV-irradiated RSV and Lipoplex(O) had no effect against RSV infection. To improve the efficacy of the RSV vaccine, we performed peptide-based epitope screening and evaluated the efficacy of the vaccine using a complex of epitope and Lipoplex(O). Two efficient B-cell epitopes were identified in nine candidate epitopes from the RSV-F protein. The vaccination with a complex of RSV-F protein epitope (F7 and F9) and Lipoplex $(\mathrm{O})$, induced a prophylactic effect on the RSVinfection based on lung histopathology and mucus clearance from the lungs. Thus, further studies on the effect of the peptide vaccine against infection by multiple RSV strains, may allow fine-tuning of a potential vaccine, and improvement of the vaccine program against RSV.


[^0]Keywords CpG-oligodeoxynucleotide • epitope • respiratory syncytial virus • vaccine

## Introduction

Respiratory syncytial virus (RSV) is a member of the Pneumovirus genus, first isolated from a chimpanzee in 1956 (Blount et al., 1956). Human RSV is the major cause of acute bronchiolitis and pneumonia, leading to a life-threatening respiratory condition in infants and young children (Harris and Werling, 2003; Kong et al., 2003; Breary and Smyth, 2007). The pathologic effect of RSV is potentially associated with viral invasion of the lower respiratory epithelium, which induces inadequate immune responses leading to cell damage and pulmonary inflammation (Katz et al., 2003; Simoes, 2003). RSV infections occur primarily within the first two years of age, and the most severe disease appears in infants from two to four months old. Recently, it has been increasingly reported that RSV also causes complications in adults, particularly in immunocompromised individuals (Culley et al., 2002; Resch et al., 2002; Deshpande and Northern, 2003; Walsh and Falsey, 2012).

Diverse studies have been undertaken to develop a prophylactic RSV vaccine. Whole-virus vaccines, such as formalin-inactivated whole-RSV vaccine (FI-RSV), have been developed conventionally, but the license of FI-RSV as an RSV vaccine was not approved after failure of its vaccine trials in the 1960s (Kim et al., 1969). The limited efficacy and safety of FI-RSV was studied, and the results showed that the FI-RSV vaccine induced Th2-type hyperimmune responses and airway hyper-responsiveness to the natural infection (Connors et al., 1992; 1994; Haynes et al., 2003; Delgado et al., 2009). Furthermore, a UV-inactivated form of RSV (UV-RSV) was also not approved as an RSV vaccine. Since then, a number of studies have been done to overcome the imperfections of FI-RSV and UV-RSV. One example of an alternative approach
involves the use of recombinant viral proteins such as Fusion (F) protein or Glycoprotein (G) of RSV (Wertz et al., 1987; Corvaïa et al., 1997; Nallet et al., 2009). To further potentiate the recombinant proteins, DNA vaccines, including adenovirus-based recombinant F (or G) expression system (Kim et al., 2010) and F (or G)-based episomal DNA vaccine (Xie et al., 2007), have been investigated as easier and quicker sources of proteins.

Investigators have reported that synthetic oligodeoxynucleotides (ODNs) containing un-methylated CpG dinucleotides flanked by specific base sequences (CpG-DNA), have potent functional effects as an immune adjuvant (Klinman et al., 2004). In previous studies, we screened the genomic DNA sequences of M. bovis with immune-modulatory activity and identified the natural phospho-diester bond CpG-DNA (namely MB-ODN 4531(O)) (Lee et al., 2006). The MB-ODN 4531(O) exhibits adjuvant activity, including the induction of antigen-dependent Th1dominated humoral immune response without severe side effects (Kim et al., 2009). We also showed that several peptides (or proteins), and MB-ODN 4531(O) encapsulated in a phosphatidyl-$\beta$-oleoyl $\gamma$-palmitoyl ethanolamine (DOPE): cholesterol hemisuccinate (CHEMS) complex (Lipoplex(O)), significantly enhanced peptide (or protein)-specific IgG production (Kim et al., 2011a; 2011b). In the present study, we evaluated the effects of vaccination with combined UV-RSV and Lipoplex(O). In addition, we identified a number of peptides from the F-protein of RSV which can induce epitope-specific antibodies by immunization with a complex consisting of epitope peptide and Lipoplex(O). We also investigated the effects of vaccination after immunization with a complex consisting of epitope peptide and Lipoplex( O ).

## Materials and Methods

Oligodeoxynucleotides. ODNs were purchased from ST Pharm Co., Ltd. (Korea). The ODNs were composed of 20 bases containing three CpG motifs (underlined: AGCAG-CGTTCGTGT CGGCCT), designated as MB-ODN. The sequence of MB-ODN 4531 contained a phospho-diester backbone (MB-ODN 4531(O)). The endotoxin content of the ODNs was $<1 \mathrm{ng} / \mathrm{mg}$ of ODN, as measured by a Limulus amebocyte assay (Whittaker Bioproducts, USA).
Cell culture. HEp-2 cells were purchased from the American Type Culture Collection (ATCC, USA). Vero cells were kindly provided by Professor Manseong Park (Korea University, Korea). The cells were grown in $150-\mathrm{mm}$ dishes with Dulbecco's modified Eagle's medium (Life Technologies, USA) containing $10 \%$ fetal bovine serum (FBS, Life Technologies), 10 mM HEPES, $100 \mathrm{U} / \mathrm{mL}$ penicillin and $100 \mathrm{mg} / \mathrm{mL}$ streptomycin.
Virus. The RSV A2 strain was kindly provided by Professor Jun Chang (Ewha Women's University, Korea). The virus was amplified on HEp-2 cells using a protocol of Prof. Chang's Laboratory. Briefly, $5 \times 10^{6} \mathrm{HEp}-2$ cells were placed in $150-\mathrm{mm}$ dishes and cultured for one day. After removal of the media, the

Table 1 Candidate epitopes of RSV F protein

| Strain | Sequences | Location Abbreviation |  |
| :---: | :--- | :--- | :--- |
|  | TSPLCTTNTKEGSNI | $218-332$ | F1 |
|  | GCDYASNKGMDTVSV | $438-452$ | F2 |
|  | LVFPSDEFDASISQV | $481-495$ | F3 |
| Human RSV | RSTPVTLSKDQLSG | $485-499$ | F4 |
| A2 strain | RSTPVTLSKDQLSGI | $553-567$ | F5 |
|  | TDRGWYCDNAGSVSF | $337-351$ | F6 |
|  | AGSVSFFPQAETCKV | $346-360$ | F7 |
|  | YGKTKCTASNKNRGII | $417-432$ | F8 |
|  | CKIMTSKTDVSSSVI | $393-407$ | F9 |
|  |  |  |  |

Candidate epitopes of hRSV A2 strain F protein were predicted with computer program (Protean in Lasergene) based on hydrophilicity, antigenic index, alpha amphipathic value, beta amphipathic value, and surface probability plot (www.dnastar.com/default.aspx).
cells were rinsed with serum-free Minimum Essential Medium (MEM, Life Technologies) and 9 mL of serum-free MEM containing 10 mM HEPES, $100 \mathrm{U} / \mathrm{mL}$ penicillin, and $100 \mathrm{mg} / \mathrm{mL}$ streptomycin were added. The cells were inoculated with virus at the MOI of 0.01 , and then incubated at $37^{\circ} \mathrm{C}$ in a $5 \% \mathrm{CO}_{2}$ incubator for 2 h with repeated rocking every $15-20 \mathrm{~min}$. Next, 21 mL of amplification media (MEM containing $6 \%$ FBS, 10 mM HEPES, $100 \mathrm{U} / \mathrm{mL}$ penicillin, and $100 \mathrm{mg} / \mathrm{mL}$ streptomycin) were added, and incubated until a cytopathic effect was observed. The virus was harvested when the cytopathic effect was over $60 \%$. The supernatants were centrifuged at $4^{\circ} \mathrm{C}$ for 1 h at 35,000 RPM. After centrifugation, the RSV virus was collected by resolubilizing the pellet with serum-free MEM. The amplified virus was titrated for infectivity using a plaque assay.
Inactivation of viral infectivity by UV exposure. UV-RSV was prepared as reported by Delgado et al. (2009) with minor modifications. Briefly, RSV A2 was irradiated by UV three times for 15 min on ice, with 204 nm of ultraviolet light. The UVinactivated virus was titrated for infectivity using a plaque assay. Selection and synthesis of peptides. The predicted B-cell epitopes of RSV F-protein were selected on the basis of their hydrophilicity values from Kyte and Doolittle (1982), surface probability values from Emini et al. (1985), $\alpha$ - and $\beta$-amphipathic values from Eisenberg et al. (1984), and antigenicity index from Jameson and Wolf (Jameson and Wolf, 1988). Nine candidatepeptides were synthesized to screen B-cell epitopes (Table 1). To identify the binding of RSV F7- and F9-epitope-specific IgGs to the homologous sequences in various RSV strains, we synthesized two typical variant sequences corresponding to the F7 and F9 epitope sequences (F7-1: $:^{346}$ AGSVSFFPLAETCKV ${ }^{360}$, F7-2. ${ }^{346} \mathrm{~A}$ GSVSFFPQADTCKV ${ }^{360}$, and F9-1: ${ }^{393}$ CKIMTSKADVSSSVI ${ }^{407}$, F9-2: ${ }^{393}$ CKIMTSKTDISSSVI ${ }^{407}$ ). The peptides were synthesized using the Fmoc solid-phase method with an automated peptide synthesizer (Peptron III-R24, Peptron, Korea), and were identified by means of a mass spectrometer (HP 1100 Series LC/MSD, Hewlett-Packard, USA).

Preparation of B-cell epitope peptide (or UV-RSV) and CpGDNA co-encapsulated in DOPE:CHEMS complexes. Liposome complexes consisting of B-cell epitope peptide of RSV F protein (or UV-RSV) and CpG-DNA co-encapsulated in DOPE:CHEMS (Lipoplex(O), were prepared as reported previously (Kim et al., 2011a; 2011b). Briefly, DOPE and CHEMS were mixed at a molar ratio of $1: 1$, and evaporated using nitrogen gas to make a solvent-free lipid film, and then resuspended with $10 \%$ ethanol in distilled water containing $250 \mu \mathrm{~g} / \mathrm{mL}$ of water soluble MB-ODN $4531(\mathrm{O})$ and $250 \mu \mathrm{~g} / \mathrm{mL}$ peptide (or $5 \times 10^{6} \mathrm{PFU}$ of UV-RSV). This process was followed by vigorous stirring at room temperature adjusting the pH to 7.0. The DOPE:CHEMS complex containing peptide and MB-ODN 4531(O) was sonicated lightly for 30 s with a sonicator (Soniifier 450, Branson Ultrasonics, USA) to achieve co-encapsulation. After the complex was filtered with a $0.22-\mu \mathrm{m}$ filter, the complex was freeze-thawed three times with liquid nitrogen. Mice and Immunization. Four-week-old female BALB/c (H-2 ${ }^{\text {b }}$ ) mice were purchased from Orient Bio (Korea) and maintained under specific pathogen-free conditions during the experiments. The mice were immunized intra-peritoneally with $200 \mu \mathrm{~L}$ of UVRSV ( $10^{6} \mathrm{PFU}$ ), a complex of each peptide and Lipoplex(O), a complex of UV-RSV and Lipoplex(O), or a mixture of MB-ODN 4531(O) (or alum) and UV-RSV, twice (or three times), at 10-day intervals. All animal procedures performed following the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research \& Quarantine Service of Korea. The protocol was permitted by Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym 2010-10, Hallym 2013-55, Hallym 2014-9, Korea).
Virus challenge experiments. RSV A2 strain ( $10^{6} \mathrm{PFU}$ ) was resuspended in $50 \mu \mathrm{~L}$ of serum-free MEM. After immunization, mice were inoculated intra-nasally with RSV A2 strain ( $10^{6} \mathrm{PFU}$ / mouse). The survival rates and body weights of the infected mice were monitored every day for 10 days. After immunization and virus challenge experiments, the mice were sacrificed under 200 $\mu \mathrm{L}$ of intra-peritoneal anesthesia (Zoletil $50 /$ Rompun ratio $=0.6 /$ 0.4 in 10 mL of distilled water), and all efforts were made to minimize pain.
Antigen-specific Ig ELISA. The amounts and titers of peptidespecific, and RSV-specific, IgG in the sera were measured by ELISA. To measure the amounts of RSV-specific total IgG, IgG1, and IgG2a; 96-well plates were coated with $2 \mu \mathrm{~g} / \mathrm{mL}$ of goat antiRSV antibody (Genway Biotech, USA) in $100 \mu \mathrm{~L}$ of $0.1 \%$ carbonate buffer overnight at $4^{\circ} \mathrm{C}$, and blocked with $100 \mu \mathrm{~L}$ Phosphate buffered saline (PBS) containing $0.1 \%$ Tween-20 (PBST) and $1 \%$ BSA. The plates were washed with PBST three times and $10^{5}$ PFU RSV A2 virus/well were added and incubated for 2 h . After washing the plates with PBST, the sera were diluted to $1: 50$ with PBS and added to the wells of each plate, followed by incubation for 2 h . The plates were washed with PBST three times and incubated with HRP-conjugated donkey anti-mouse IgG antibody, anti-mouse IgG1 antibody, or anti-mouse gG2a antibody (The Jackson Laboratory, USA) (ratio $1: 5000$ ) for 1 h .

The plates were washed with PBST three times, and PBS one time, and treated with $100 \mu \mathrm{~L}$ tetramethylbenzidine (TMB) peroxidase substrate (KPL, USA). The reaction was stopped with $100 \mu \mathrm{~L}$ TMB-stop solution (KPL), and the plates were analyzed at 450 nm by a Spectra Max 250 micro-plate reader (Molecular Devices, USA).

To determine the amounts and titers of peptide-specific IgG, 96well plates were coated with $50 \mu \mathrm{~g} / \mathrm{mL}$ of each peptide in $100 \mu \mathrm{~L}$ of $0.1 \%$ carbonate buffer overnight at $4^{\circ} \mathrm{C}$. After coating, the plates were blocked with PBST containing $1 \%$ BSA for 1 h and then washed with PBST three times. The sera were diluted to 1:50 with PBS, added to the wells of each plate, and then incubated for 2 h . Total IgG, IgG1, and IgG2a levels were measured as described above.
Histology. To identify the histology of lung injury, the specimens were stained with hematoxylin and eosin using standard procedures. Briefly, mouse lungs were fixed with 10 mL of $10 \%$ neutral formalin solution for 4-5 days. After dehydration of specimens using serially-diluted ethanol, the specimens were treated with xylene to achieve paraffin embedding. Subsequently, the specimens were cut into $5-\mu \mathrm{m}$-thick sections, and the paraffin was removed with xylene. The specimens were stained with Gill's Hematoxylin V (Muto Pure chemicals LTD, Japan) for 5-10 min and washed with flowing tap water. Next, the specimens were stained with alcoholic Eosin-Y solution (Sigma-Aldrich, USA) for 5 min and dehydrated by serially-diluted ethanol and fixed with Malinol mounting solution (Muto Pure chemicals LTD). All images were observed with a Nikon Eclipse E-200 microscope (Nikon, Japan). Periodic acid Schiff (PAS) staining. To identify the mucus expression in lung injury, the specimens were stained with PAS using the standard procedures. Briefly, paraffin embedded specimens were cut into $5 \mu \mathrm{~m}$-thick sections, and the paraffin was removed with xylene. The specimens were incubated in periodic acid (Sigma-Aldrich) for 10 min . After rinsing with water, the specimens were stained with Schiff reagent (Sigma-Aldrich) for 15 min , and then rinsed with sulfite rinse solution (containing $10 \%$ sodium meta-bisulfite, 1 N HCl ) and water. For counter staining, the specimens were stained with hematoxylin and fixed with mounting solution.

## Results

Adjuvant activity of Lipoplex(O) in RSV vaccination. Inactivated RSV vaccines such as UV-RSV and FI-RSV failed to receive licenses, because the inactivated RSV induced Th2-type hyper-immune responses and produced non-protective antibody (Kim et al., 1969). Previously, we showed that Lipoplex(O) induces Th1-dominated humoral immune response and enhances the production of IgG 2 a specific to antigenic protein in mice (Kim et al., 2011a; 2011b). Therefore, we compared the abilities of the adjuvant activity of CpG-DNA (MB-ODN 4531(O)), Lipoplex(O), and alum to induce the vaccination effects of UV-inactivated RSV.


Fig. 1 Comparison of adjuvants capability in the vaccination of UV-RSV. (A) Experimental schedule of UV-RSV immunization. To compare the abilities of the adjuvants (CpG-DNA (MB-ODN 4531(O)), Lipoplex(O) and alum), BALB/c mice ( $\mathrm{n}=10 /$ group) were injected intra-peritoneally twice with the complex of UV-RSV and each adjuvant. After 10 days, the immunized mice were challenged intra-nasally with the RSV A2 strain ( $10^{6} \mathrm{PFU} /$ mouse). (B) Induction of a strong serologic response to RSV infection by vaccination with the complex of UV-RSV and each adjuvant. The antisera were collected 10 days after the virus challenge, and then amounts of RSV-specific total IgG, IgG1, and IgG2a were assayed by ELISA. (C) Measurement of body weight changes after the virus challenge.

To determine the adjuvant effects of Lipoplex (O) on RSV vaccination, a complex of UV-RSV and Lipoplex(O) was prepared. To evaluate the prophylactic efficacy of vaccines containing adjuvants such as MB-ODN 4531(O), Lipoplex(O), or alum; the BALB/c mice were challenged with RSV 10 days after vaccination (Fig. 1A). First, we confirmed that RSV-specific total IgG was produced in the mouse by RSV vaccination. When mice were challenged with RSV, RSV-specific total IgG was significantly produced in an adjuvant-independent manner. However, immunization with a mixture of UV-RSV and MB-ODN 4531(O), or a complex of UV-RSV and Lipoplex(O), produced a much higher level of $\operatorname{IgG} 2 \mathrm{a}$ than $\mathrm{IgG1}$ in the mice. These results suggest the induction of a Th1-dominated humoral response in the animal experiments when MB-ODN 4531(O) or Lipoplex(O) was applied as an adjuvant (Fig. 1B). We also found that a larger amount of RSV-specific IgG1 was produced in the mice immunized with a mixture of UV-RSV and alum. However, alum was a less potent adjuvant than MB-ODN 4531(O) and Lipoplex(O) for induction of a Th1-dominated humoral response (Fig. 1B).

All the mice immunized with UV-RSV vaccines containing adjuvants such as MB-ODN 4531(O), Lipoplex(O), or alum showed a $100 \%$ survival rate (data not shown) and had regained weight 10 days after the virus challenge (Fig. 1C). We also examined the lung histopathology of RSV-infected mice 5 and 10 days after infection, and found pulmonary lesions consisting of severe perivascular and peri-bronchiolar lymphocytic infiltrates. The mice immunized with UV-RSV vaccines containing adjuvants such as MB-ODN 4531(O), Lipoplex(O) or alum had the same lymphocytic infiltrates in the lung by RSV infection, suggesting that there was no prominent effect of the RSV vaccines against RSV infection.
Selection of B-cell epitopes from RSV F-protein and induction of epitope-specific IgG production by a complex of epitope and Lipoplex(O). Whole-RSV vaccines, such as the UV-RSV vaccine, did not moderate the symptoms of RSV infection even if Thl-dominated humoral response was induced by adjuvants such as MB-ODN 4531(O) and Lipoplex(O) (Figs. 1 and 2). Therefore, we used a peptide-vaccine system to improve the efficacy of the RSV vaccine by inducing the Th1 immune response and epitopespecific antibody.

The RSV F fusion protein is an important surface protein, triggering membrane fusion and entry of the virus into the cell cytoplasm as well as allowing the formation of syncytia (Melero, 2007). To select a B-cell epitope from the F-protein of the RSVA strain Long, we synthesized nine candidate peptide sequences on the basis of their hydrophilicity, hydrophobicity, secondary structure, and antigenicity index as described in "Materials and Methods" (Table 1), and then prepared a complex containing each epitope and Lipoplex $(\mathrm{O})$. When BALB/c mice were immunized with the complex of each epitope and Lipoplex(O), the BALB/c mice induced peptide (F7 or F9)-specific IgG production more


Fig. 2 Effects of UV-RSV vaccination on macroscopic appearance and histopathology of lungs. Immunization and virus challenge experiments were performed as described in Fig. 1. (A) Macroscopic appearance of lungs from RSV-challenged mice. Macroscopic appearance was monitored at 5 and 10 days after the virus challenge. (B) Histopathology of the lungs. The lungs were collected 5 and 10 days after the virus challenge. The lung sections were stained with hematoxylin and eosin. Scale bars: 40 X column, $250 \mu \mathrm{~m} ; 200 \mathrm{X}$ column, $50 \mu \mathrm{~m}$.
markedly than did the control mice (Fig. 3A). Immunization with a complex of F7 (or F9) epitope and Lipoplex(O), produced a much higher level of IgG2a, which is involved in the induction of a Th1-dominated humoral response upon vaccination (Fig. 3B), than IgG1 in the mice. These two epitopes (F7 and F9) overlap with one of the membrane fusion sites (Fig. 3C).

These results suggest that the complex of B-cell epitope and Lipoplex $(\mathrm{O})$ induced epitope-specific IgG production, and that Lipoplex $(\mathrm{O})$ is important for producing an epitope-specific Th1-
dominant humoral immune response.
Binding-reactivity of IgG produced by a complex of epitope and Lipoplex(O). When we analyzed the selected B-cell epitopes (F7 and F9) and corresponding sequences in the various RSV strains, the selected B-cell epitopes from the F-protein of the RSVA strain Long had the highest consensus sequences among the Fproteins of various RSV strains. The F7 epitope (AGSVSFFPQ AETCKV) is highly conserved among RSV strains reported to date, with only one amino acid difference (F7-1, AGSVSFFPL


Fig. 3 B cell epitope selection from the F protein of RSV. (A, B) BALB/c mice ( $\mathrm{n}=5 /$ group) were immunized with the complex of each candidate epitope and Lipoplex $(O)$ three times, at 10-day intervals. The antisera were collected, and the amounts of each peptide-specific total IgG (A), and each peptide-specific IgG1 and IgG2a (B), were assayed by ELISA. (C, D) Location of F7 epitope and F9 epitope in the three-dimensional structure of the RSV F-protein. Red: F7 epitope; Green: F9 epitope. Structures of monomer F-protein (C) and homo-trimer F-protein (D), were depicted by computer analysis. The structural image of F-protein from the RSV-A2 strain was created with the use of PyMOL (www.pymol.org), and information about the F-protein structure was obtained from the Protein Data Bank (PDB: 1JSM).

AETCKV; F7-2, AGSVSFFPQADTCKV). The F9 epitope (CKIMTSKTDVSSSVI) is also highly conserved among reported RSV strains with only one amino acid difference (F9-1, CKIMTS K_્ADVSSSVI; F9-2, CKIMTSKTDISSSVI). We also immunized BALB/c mice with the complex of each corresponding epitope (F7-1, F7-2, F9-1, or F9-2) and Lipoplex(O). Interestingly, the BALB/c mice produced each epitope-specific IgG more markedly than did the control mice (Fig. 4).

We next investigated whether the epitope-specific antibodies produced by the complex of epitope and Lipoplex $(\mathrm{O})$ could bind with each corresponding epitope. The antisera from the mice immunized with the F7 epitope efficiently bound to F7-1 and F72 epitopes, but the antisera from the mice immunized with the F71 epitope could not bind to F7. The antisera against F9 (F9-1 or F9-2) showed specific binding activity to all F9 epitopes (Fig. 4). Prophylactic efficacy of a vaccine containing epitope and Lipoplex( $\mathbf{O}$ ) complex against an RSV infection in mice. To evaluate the prophylactic efficacy of a vaccine containing a complex of F-protein epitope and Lipoplex(O), we challenged the

BALB/c mice with the RSV 10 days after vaccination (Fig. 5A). Two groups of mice were immunized with a complex of F7 epitope and Lipoplex(O) (F7+Lipoplex(O)) or with a complex of F9 epitope and Lipoplex(O) (F9+Lipoplex(O)). The body weight of RSV-challenged mice decreased until 5 days after virus infection and regained after 10 days. All RSV-challenged mice after vaccination also regained weight after 10 days (Fig. 5B). To gain additional information regarding the immunogenic potential of the vaccine containing a complex of epitope peptide and Lipoplex(O), we obtained sera from the immunized mice 10 days after the virus challenge. The sera from the mice immunized with a complex of RSV F-protein epitopes ( F7 epitope or F9 epitope) showed a much higher level of IgG2a than IgG1 in the mice, relating to the induction of a Th1-dominated humoral response in the vaccination experiments (Fig. 5C). Next, we observed the lung histopathology of RSV-infected mice 5 and 10 days after the RSV challenge. Lungs from control RSV-infected mice showed lesions consisting of typical perivascular and peri-bronchiolar lymphocytic infiltrates, and more mucus in the lungs. However, the mice


Fig. 4 Cross-reactivity of antisera obtained by the complex of F7 epitope (or F9 epitope) and Lipoplex(O) to its corresponding sequences in various RSV strains. The antisera were collected 10 days after the final immunization, and then titers of each peptide-specific IgG were measured by ELISA. (A-C) The antisera were obtained from BALB/c mice immunized with the complex of F7 epitope (F7-1 epitope or F7-2 epitope) and Lipoplex(O). Each antiserum was added to plates coated with F7 epitope (A), F7-1 epitope (B) or F7-2 epitope (C). (D-F) The antisera were obtained from BALB/c mice immunized with the complex of F9 epitope (F9-1 epitope or F9-2 epitope) and Lipoplex(O). Each antiserum was added to plates coated with F7 epitope (D), F7-1 epitope (E), or F7-2 epitope (F).
immunized with a complex of RSV F-protein epitope (F7 or F9) and Lipoplex $(\mathrm{O})$, had moderate lymphocytic infiltrates and reduced mucus accumulation in their lungs after the RSV challenge (Fig. 6).

## Discussion

Adjuvant activities of CpG-DNA have gained increased attention as a potentially useful strategy for the induction of Ag-driven Th1 responses (Klinman et al., 2004). Phosphorothioate backbonemodified CpG-DNAs (PS-DNA) have a sulfur substitution instead of non-bridging oxygens in the backbone to provide the nuclease resistance of CpG-DNA. The PS-DNA has been studied for therapeutic applications (Broide et al., 1998; Zimmermann et al., 1998; Ballas et al., 2001). However, several studies reported the
initiation of phosphorothioate backbone-related side effects such as transient splenomegaly, lymphoid follicle destruction, and arthritis by PS-DNA stimulation (Deng et al., 1999; Sparwasser et al., 1999; Heikenwalder et al., 2004). To overcome the side effects of CpG-DNA, sequence rearrangement and backbone modification of PS-DNA were investigated to reduce severe side effects. Previously, we identified the natural phosphodiester bond CpGDNA, which modulates TLR9-mediated innate immune responses without severe side effects (Kim et al., 2009). In contrast to PSDNA, PO-DNA shows poor activity in human cells. To improve the functional effects of PO-DNA, we investigated the effects of liposome-encapsulated PO-DNA. After stimulation with PODNA encapsulated in liposomes such as DOPE:CHEMS complex (Lipoplex(O), the production of cytokines including IL-6, IL-12, and IFN- $\gamma$, was significantly induced (Kim et al., 2011a). We also showed that the adjuvant effect of Lipoplex(O): protein antigen-


Fig. 5 Production of RSV-specific IgG by the complex of F-protein B-cell epitope and Lipoplex(O). (A) Vaccination schedule. Each complex of Fprotein B-cell epitope and Lipoplex $(\mathrm{O})$ were immunized to BALB/c mice $(\mathrm{n}=10)$ three times at 10 -day intervals. Ten days after the third immunization, the mice were challenged intra-nasally with the RSV-A2 strain ( $10^{6} \mathrm{PFU} /$ mouse). (B) Measurement of body-weight changes after the virus challenge. (C) Induction of a strong serologic response to RSV infection by vaccination with the complex of each epitope and Lipoplex(O). The antisera were collected 10 days after the virus challenge, and the amounts of RSV-specific IgG were assayed by ELISA. ${ }^{*} p<0.005 ;{ }^{* *} p<0.05$.
specific specific IgG2a was produced more than that of IgG1 (Kim et al., 2011a; 2011c). Furthermore, we determined that a complex of peptide and Lipoplex $(\mathrm{O})$ without carriers significantly enhanced peptide-specific IgG production and Th1-dominated protective immune response (Kim et al., 2011a; 2011b). Our results were successfully applied to improve peptide vaccine efficacy in vaccination against influenza-A virus and hepatocellular carcinoma (Rhee et al., 2012; Kwon et al., 2012; Kwon et al., 2013).

It is difficult to develop RSV vaccines, because FI-RSV and UV-RSV induce Th2-type hyper-immune responses. Therefore, no whole-RSV vaccine has been licensed yet. To overcome this limitation, we used Lipoplex $(\mathrm{O})$ as an adjuvant to increase the Thl-immune response. However, vaccination with a complex of UV-RSV and Lipoplex $(\mathrm{O})$ had no effect against RSV infection (Figs. 1 and 2). In another strategy to improve the efficacy of the RSV vaccine, we performed peptide-based epitope screening and attempted to develop an RSV-peptide vaccine using a complex of epitope and Lipoplex(O). We successfully identified two B-cell epitopes, F7 and F9, out of nine candidate epitopes from the RSV F-protein, by using a complex of each predicted epitope and Lipoplex(O) (Fig. 3). Vaccination with a complex of RSV Fprotein epitope (F7 and F9) and Lipoplex(O), resulted in
prophylactic effects (such as improved lung histopathology and mucus clearance from the lungs) upon RSV infection (Figs. 5 and 6). Thus, it is clear that this peptide vaccine is better than wholeRSV vaccine. This could be the result from a specific immune response without the side effects, which accompany vaccination with whole-RSV vaccine. Further studies on the prophylactic effect of the complex of F-protein epitope and Lipoplex(O) on multiple RSV strains should allow fine tuning of the peptidebased vaccine as a potential universal vaccine for RSV. These efforts, in turn, should lead to an improved vaccine program against RSV in the future.

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Fig. 6 Effects of the complex of F-protein B-cell epitope and Lipoplex $(\mathrm{O})$ vaccination on macroscopic appearance and histopathology of mouse lungs. Immunization and virus challenge experiments were performed as described in Fig. 5. (A, B) Macroscopic appearance and histopathology of the lungs of RSV-challenged mice. Macroscopic appearance and histopathology of lungs were monitored 5 or 10 days after the virus challenge. The lungs were collected 5 or 10 days after the virus challenge. The lung sections were stained with hematoxylin and eosin (A) and PAS (B). Scale bars: 40X column, $250 \mu \mathrm{~m} ; 200 \mathrm{X}$ column, $50 \mu \mathrm{~m} ; 400 \mathrm{X}$ column, $20 \mu \mathrm{~m}$.

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