Recombinant baculovirus-based vaccine expressing M2 protein induces protective CD8⁺ T-cell immunity against respiratory syncytial virus infection

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Respiratory syncytial virus (RSV) is an important cause of acute lower respiratory tract disease in infants, young children, immunocompromised individuals, and the elderly. However, despite ongoing efforts to develop an RSV vaccine, there is still no authorized RSV vaccine for humans. Baculovirus has attracted attention as a vaccine vector because of its ability to induce a high level of humoral and cellular immunity, low cytotoxicity against various antigens, and biological safety for humans. In this study, we constructed a recombinant baculovirus-based vaccine expressing the M2 protein of RSV under the control of cytomegalovirus promoter (Bac_RSVM2) to induce CD8⁺ T-cell responses which play an important role in viral clearance, and investigated its protective efficacy against RSV infection. Immunization with Bac_RSVM2 via intranasal or intramuscular route effectively elicited the specific CD8⁺ T-cell responses. Most notably, immunization with Bac_RSVM2 vaccine almost completely protected mice from RSV challenge without vaccine-enhanced immunopathology. In conclusion, these results suggest that Bac_RSVM2 vaccine employing the baculovirus delivery platform has promising potential to be developed as a safe and novel RSV vaccine that provides protection against RSV infection.

Keywords: recombinant baculovirus, respiratory syncytial virus, m2 protein, cd8 t cells, vaccine

Introduction

Respiratory syncytial virus (RSV) causes acute lower respiratory tract infection in infants, young children, immunocompromised individuals, and the elderly (Welliver, 2003; Falsey, 2005). Formalin-inactivated RSV (FI-RSV) vaccine was the first attempted RSV vaccine candidate in the 1960s, but in clinical trials, this vaccine elicited Th2-type immunopathology following natural RSV infection (Kapikian *et al.*, 1969; Kim *et al.*, 1969). Also, several subsequent studies indicated that recombinant vaccinia virus expressing RSV G

protein (vvG), as FI-RSV did, exhibited immunopathological problems such as pulmonary eosinophilia by enhancing Th2 skewing of the CD4⁺ T-cell response (Openshaw *et al.*, 1992; Bembridge *et al.*, 1998; Dudas and Karron, 1998; Johnson *et al.*, 2001; Castilow *et al.*, 2007). These reports show that CD4⁺ T-cell immune responses are important for the elimination of RSV (Graham *et al.*, 1991; Plotnicky-Gilquin *et al.*, 2000), but excessive immune responses can lead to immunopathology (Alwan *et al.*, 1992).

Meanwhile, development of RSV vaccine using CD8⁺ T-cell responses has also been studied. Generally, it is known that cytotoxic activity of CD8⁺ T-cells can suppress viral replication and mediate pathogen clearance. RSV M2 gene encodes both M2-1 (22 kDa) and M2-2 (11 kDa) proteins by two overlapping open reading frames (ORFs) (Collins and Wertz, 1985; Collins *et al.*, 1990) and highly immunodominant H-2K^d restricted epitope is located in M2-1 protein (Openshaw *et al.*, 1990; Kulkarni *et al.*, 1995). So the RSV M2-1 could be an excellent target of major histocompatibility complex (MHC) Class I-restricted cytotoxic T lymphocytes (CTL), and elicit RSV-specific cellular immune responses leading to viral clearance during RSV infection (Connors *et al.*, 1992; Kulkarni *et al.*, 1993). Although robust CD8⁺ T-cell responses caused body weight loss and lung pathology in RSV infected mice (Cannon *et al.*, 1988; Ostler *et al.*, 2002; Ruckwardt *et al.*, 2010), several studies demonstrated that RSV M2-specific CD8⁺ T-cell responses inhibited vaccine-enhanced Th2-driven immunopathology during RSV infection (Hussell *et al.*, 1997; Srikiatkachorn and Braciale, 1997; Olson and Varga, 2007). In addition, Lee *et al.* (2014) recently demonstrated that administration of RSV vaccine containing CD8⁺ T-cell epitope with CD137-activating adjuvant could provide protective immunity and reduce RSV morbidity and mortality in aged mice. For these reasons, the CD8⁺ T-cell-based vaccine using the RSV M2 protein could be a potential RSV vaccine candidate.

Baculovirus has attracted attention as a vaccine vector due to the following characteristics. Baculovirus is a double stranded DNA (dsDNA) virus, and it mainly infects insect cells. Interestingly, baculovirus cannot naturally replicate in mammalian cells because transcriptional gene silencing occurs when baculovirus infects non-permissive cells (He *et al.*, 2009). Above all, humans do not possess pre-existing anti-baculovirus immune responses (Luo *et al.*, 2013), so the problem of pre-existing immunity faced by human-derived viral vectors can be avoided. Based on these features, baculovirus is expected to be safe for humans as a vaccine vector. Moreover, baculovirus can express foreign proteins in mammalian cells

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using promoters/enhancers such as CMV, CAG, SV40 and HBV (Kost and Condreay, 2002; Kost *et al.*, 2005). Recombinant baculovirus carrying foreign genes induces a high level of humoral/cellular immunity and has low cytotoxicity against various antigens (Madhan *et al.*, 2010). Besides, unlike subunit vaccines that require adjuvants, baculovirus alone can act as an adjuvant because it can promote humoral and cellular immunity by itself (Kost *et al.*, 2005; Chen *et al.*, 2011; Lu *et al.*, 2012). Thus, baculovirus may present a new direction in the development of a vector vaccine that is different from the existing strategies.

Previously, we reported that a recombinant baculovirus vaccine displaying RSV G protein (Bac-RSV/G) induced protective immune responses against RSV A challenge without vaccine-enhanced immunopathology (Kim and Chang, 2012). Also, we recently demonstrated that a recombinant baculovirus-based vaccine displaying the hemagglutinin (HA) of influenza virus provided broad cross-protective immunity against both homologous and heterologous strains of influenza virus (Sim *et al.*, 2016). Based on these studies, we attempted to develop a RSV vaccine that induces CD8⁺ T-cell responses using a baculovirus platform in this study. We constructed a recombinant baculovirus containing RSV M2 gene under the control of CMV promoter (Bac_RSVM2) and investigated protective cellular immune responses against RSV infection. This study demonstrated that RSV-specific CD8⁺ T-cell responses—especially, the production of RSV M2₈₂₋₉₀-specific IFN- γ by effector CD8⁺ T cells—were induced in mice immunized with Bac_RSVM2 through intranasal and intramuscular administrations. Also, Bac_RSVM2 vaccine conferred protection from RSV challenge without vaccine-induced immunopathology and systemic diseases, suggesting that Bac_RSVM2 is a potential vaccine candidate for RSV infection.

Materials and Methods

Generation of recombinant baculovirus expressing RSV M2 protein

To generate pFastBac1-CMV plasmid, the pCI-neo vector (Promega) was cut with *Bgl*II and *Not* I to obtain CMV fragment. The pFastBac1 vector (Invitrogen) was also cut with the same restriction enzymes (*Bam*HI and *Not*I), and then the CMV fragment was inserted into the pFastBac1-vector. Next, to construct pFastBac1-CMV/RSVM2 plasmid, RSV M2 gene was amplified by PCR with the forward primer (5'-AAG AAT TCA TGT CAC GAA GGA ATC CTT G-3') and the reverse primer (5'-GGG CGG CCG CTC AGG TAG TAT CAT T-3') using RSV A2 cDNA as a template. The amplified PCR product was inserted into the pFastBac1-CMV plasmid, which was previously digested with *Eco*RI and *Not*I (pFastBac1-CMV/RSVM2). Lastly, this expression cassette was inserted into the baculovirus genome within DH10BacTM (Invitrogen) through specific transposition sites according to the manufacturer's protocol of the Bac-to-Bac system (Invitrogen). The rBac-RSVM2 plasmid was transfected into Sf-9 cells. The recombinant virus was harvested at 96 h post-transfection, and then amplified following manufacturer's protocol.

Cell lines and preparations of RSV and recombinant baculovirus stock

Recombinant baculoviruses were propagated in *Spodoptera frugiperda* 9 (Sf-9) insect cells (Invitrogen) in SF-900II serum-free medium (Gibco BRL) at 28°C. To obtain the recombinant virus stock, final culture supernatants of infected Sf-9 cells were harvested and purified through sucrose cushion (25% w/w sucrose in 5 mM NaCl, 10 mM EDTA in PBS) centrifugation (24,000 rpm for 75 min at 4°C). The pellets were resuspended in 0.25 M sucrose and stored in LN₂. The viral titer of the purified recombinant baculovirus was measured by plaque assay in Sf-9 cells following the manufacturer's protocol (Invitrogen). To obtain the RSV A2 strain, HEp-2 (ATCC) cells were cultured in minimal essential medium (MEM, Hyclone) containing 10% fetal bovine serum (FBS), and RSV A2 stock was propagated in HEp-2 cells as previously described (Lee and Chang, 2017). All virus titrations were performed by standard plaque assay.

Mice and ethics statement

6-week-old female BALB/cAnNCrljOri mice were purchased from Orient Bio Inc. and cared under specific pathogen-free (SPF) conditions. To minimize stress and alarm, mice were monitored daily and handled gently. All animal experiments were performed with four mice per group ($n = 4/\text{group}$) and conducted in accordance with the suggestions in the Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals. All animal studies were approved by the guidelines of Ewha Womans University Institutional Animal Care and Use Committee (IACUC-16-056).

Immunization and RSV challenge

6-week-old female mice ($n = 4/\text{group}$) were immunized with PBS, Bac_control (10^7 PFU/mouse), or Bac_RSVM2 (10^7 PFU/mouse) via intranasal (IN) or intramuscular (IM) route. For IN immunizations, mice were anesthetized by isoflurane (Ifran[®]; Hana Pharm) inhalation, and PBS (50 μ l) and vaccines (in a volume of 50 μ l) were delivered to the left nostril route once. For IM immunizations, mice were injected once with vaccines (in a volume of 100 μ l) in the hind-right leg. At 28 days after the last immunization, all mice were challenged with RSV A2 (10^6 PFU in 50 μ l) through IN route, and body weight loss of the challenged mice was monitored daily for 5 days.

Sample preparation

Five days after RSV challenge, mice were sacrificed by CO₂ euthanasia, and bronchoalveolar lavage (BAL) fluids were obtained by washing the airways with 1 ml of PBS. BAL cells and supernatants were separated by centrifugation, and the BAL cells were used to measure leukocyte recruitment. Lung tissues obtained from the immunized mice were homogenized and passed through a 70 μ m cell strainer (SPL). Lung mononuclear cells and supernatants were separated by centrifugation to measure immune cell proportion and viral replication in the lungs.

Flow cytometric analysis

To measure the population of granulocytes in the BAL, BAL cells obtained from the immunized mice were incubated with rat anti-mouse CD16/CD32 (BD Biosciences) blocking antibody for 10 min at RT, and stained with anti-Siglec-F (E50-2440, BD Biosciences), anti-Gr-1 (RB6-8C5, BioLegend) anti-CD11c (N418, eBioscience), and anti-CD45 (30-F11, BioLegend) antibodies for 30 min at 4°C in dark. For tetramer analysis, lymphocytes obtained from lung tissue were blocked with anti-mouse CD16/32 (BD Biosciences) antibody and stained with anti-CD8 (53-6.7, BioLegend) antibody, anti-CD44 (IM7, BioLegend) antibody, and MHC class I H-2K^d tetramer complexes loaded with RSV M2₈₂₋₉₀ (H-2K^d/SYIGSINNI) for 30 min at 4°C in dark. After antibody staining, all the stained cells were fixed with FACS lysing solution (BD Biosciences) and analyzed by BD LSR Fortessa (BD Biosciences). To investigate the cytokine-producing cells, lung lymphocytes were resuspended in IMDM supplemented with 10% heat-inactivated FBS (10% IMDM) and stimulated with 50 µg/ml phorbol myristate acetate (PMA, 1:1,000), 500 µg/ml ionomycin (1:1,000), or 10 µM RSV M2(82-90) peptide in 10% IMDM containing 10 ng of recombinant human IL-2 (BioLegend) and Brefeldin A (1:1,000, eBioscience) at 37°C for 5 h in dark. After stimulation, these cells were surface stained with anti-CD8 antibody (53-6.7) and anti-CD44 antibody (IM7) for 30 min at 4°C, and then fixed. To stain the intracellular cytokines, fixed cells were permeabilized with FACS buffer (0.5% FBS, 0.09% NaN₃ in PBS) containing 0.5% saponin (Sigma-Aldrich) for 15 min at RT and stained with anti-IFN-γ (XMG 1.2) antibody for 30 min at RT. The stained cells were then analyzed by BD LSR Fortessa (BD Biosciences). All flow cytometry data was analyzed using Flowjo software (TreeStar Inc.).

Lung viral titration

Five days after RSV challenge, all mice were sacrificed by CO₂ euthanasia, and lung single-cell suspensions were obtained by processing through a 70 µm cell strainer (SPL) into serum-

free MEM. The supernatants were collected by centrifugation, and lung viral titer was determined by plaque assay in HEp-2 cells using the supernatants. Viral titers are expressed as PFU/g of lung, and the limit of detection is 100 PFU/g of lung.

Statistical analysis

All values in this study are expressed as mean ± standard deviation (SD). To determine statistically significant differences between two groups, Student's *t*-test was used, and a *P*-value less than 0.05 was taken to indicate statistical significance (**P* < 0.05, compared with PBS; ***P* < 0.05, compared with Bac_RSVM2 IN).

Results

Generation of recombinant baculovirus-based RSV M2 vaccine and experimental design

To generate a recombinant baculovirus-based RSV M2 vaccine candidate, we used the Bac-to-Bac expression system with a pFastBac1 vector. We constructed a pFastBac1 vector containing the amino acid sequence of RSV M2 under the control of Cytomegalovirus (CMV) immediate-early promoter as shown in Fig. 1A. The CMV promoter is generally used to induce gene expression in mammalian cells (Kost and Condreay, 2002). We next planned the animal experiment to confirm that the Bac_RSVM2 has efficacy as a RSV vaccine (Fig. 1B). BALB/c mice were immunized via intranasal (IN, in order to induce mucosal immune responses) or intramuscular (IM, in order to induce systemic immune responses) route with 10⁷ PFU/mouse of either Bac_RSVM2 or Bac_control (Fig. 1B). The Bac_control-immunized group was used as a control, and the PBS group was used as a negative control; exceptionally, the PBS group was administered only via IN route. At 5 days post-RSV challenge, the mice were sacrificed to investigate cellular immune responses induced by Bac_RSVM2 and protective efficacy of the vaccine.

Fig. 1. Construction of recombinant Bac_RSVM2 vaccine and timeline of animal experiment. (A) Fig. 1A shows a schematic diagram of the recombinant baculovirus-based RSV M2 vaccine candidate. A pFastBac1-CMV/RSVM2 (Bac_RSVM2) was constructed using Bac-to-Bac baculovirus expression system as described in the Materials and Methods. (B) Experimental scheme used for animal experiments is shown in Fig. 1B. In the intranasal (IN) immunization, mice were vaccinated once with PBS, Bac_control, or Bac_RSVM2. In the intramuscular (IM) immunization, mice were vaccinated once with the same antigen doses, except for PBS.

RSV M2-specific CD8⁺ T-cell responses in mice immunized with Bac_RSVM2

CD8⁺ T cells play an important role in viral clearance and inhibition of Th2-type cell expansion in experimental RSV models (Hussell *et al.*, 1997; Srikiatkachorn and Braciale, 1997). To investigate cellular immune responses induced by Bac_RSVM2, we harvested the lung cells from all of the immunized mice at 5 days post-challenge, and the lung cells were stained with H-2K^d-M2₈₂₋₉₀ (SYIGSINNI) tetramer to quantify RSV M2-specific CD8⁺ T cell population (Fig. 2). For both IN and IM routes, immunization with Bac_RSVM2 elicited the RSV M2-specific CD8⁺ T cells in mice (Fig. 2A and B). Interestingly, the percentage of RSV M2-specific CD8⁺ T cells was significantly higher in Bac_RSVM2 IM group (~50% of CD8⁺ T cells on average) than in Bac_RSVM2 IN group (~25% of CD8⁺ T cells on average, Fig. 2B, lower panel). However, the absolute number of RSV M2-specific CD8⁺ T cells was not significantly different between Bac_RSVM2 IN and Bac_RSVM2 IM groups (Fig. 2B, upper panel). In the PBS and Bac_control groups, RSV M2-specific CD8⁺ T cells were slightly induced (about 15% and 13% of CD8⁺ T cells on average, respectively), which might be the primary M2-specific CD8⁺ T-cell responses induced by RSV

challenge (Fig. 2B, lower panel). These results indicate that Bac_RSVM2 immunization via IN or IM route can prime RSV M2-specific CD8⁺ T cells and induce the recall responses in the lungs following RSV challenge.

RSV M2₈₂₋₉₀-specific IFN- γ -producing CD8⁺ T cells are boosted by Bac_RSVM2 immunization

IFN- γ produced by CD8⁺ T cells is known as an antiviral cytokine and is important for host defense and cell-mediated immune responses against viral pathogens. To investigate the level of effector CD8⁺ T cells producing IFN- γ , we performed intracellular cytokine staining (ICS) assay in the lung cells obtained from all the immunized mice at 5 days post-challenge. As shown in Fig. 3, when the lung cells were stimulated with RSV M2₈₂₋₉₀ peptide (SYIGSINNI), significantly more RSV M2₈₂₋₉₀-specific, IFN- γ -producing CD8⁺ T cells could be detected in the lung cells from Bac_RSVM2-immunized mice, compared with the lung cells from the mice immunized with PBS or Bac_control (Fig. 3A and B). More specifically, RSV M2₈₂₋₉₀-specific IFN- γ -producing CD8⁺ T cells accounted for 11% of CD8⁺ T cells on average in the Bac_RSVM2 IN group, and RSV M2₈₂₋₉₀-specific IFN- γ ⁺ CD8⁺ T cells accounted for 15% of CD8⁺ T cells on aver-

Fig. 2. RSV M2-specific CD8⁺ T-cell responses in mice immunized with Bac_RSVM2. Five days after RSV A2 challenge, lung mononuclear cells obtained from the vaccinated mice were stained with anti-CD8 antibody, anti-CD44 antibody, and H-2K^d-M2₈₂₋₉₀ tetramer, and flow cytometry analysis was performed. (A) Cells gated for CD8⁺ T cells are shown in zebra plots, and (B) the number (upper panel) and percentage (lower panel) represent the frequency of RSV M2-specific CD8⁺ CD44⁺ T cells. Data are presented as mean $\pm$ SD ($n = 4$ /group), and significant differences are marked with asterisks. * $P < 0.05$, compared with PBS; ** $P < 0.05$, compared with Bac_RSVM2 IN.

Fig. 3. RSV M2₈₂₋₉₀-specific IFN- γ -producing CD8⁺ T-cell responses induced by Bac_RSVM2. Five days after RSV A2 challenge, lung mononuclear cells were harvested and stimulated with/without RSV M2 82-90 peptide (SYIGSINNI) for 5 h *in vitro*. The stimulated lung cells were stained with anti-CD8 antibody and anti-CD44 antibody and fixed. After permeabilization, the cells were stained using anti-IFN- γ antibody and analyzed by flow cytometry. (A) Cells gated for CD8⁺ T cells are shown in contour plots, and (B) the number (upper panel) and percentage (lower panel) represent the frequency of RSV M2₈₂₋₉₀-specific, IFN- γ -producing CD8⁺ T cells. Data are presented as mean $\pm$ SD ($n = 4$ /group). Significant differences from the PBS are marked with asterisks. * $P < 0.05$.

age in the Bac_RSVM2 IM group (Fig. 3B, lower panel). The absolute number of RSV M2₈₂₋₉₀-specific IFN- γ -producing CD8⁺ T cells shows significant difference between IN and IM groups ($P = 0.05$, Fig. 3B, upper panel). In the control groups, when the lung cells were stimulated with RSV M2₈₂₋₉₀ peptide, the number and percentage of RSV M2₈₂₋₉₀-specific IFN- γ -producing CD8⁺ T cells slightly increased in both PBS and Bac_control groups, which might be primary M2-specific responses upon RSV infection (Fig. 3B). Taken together, these results indicate that immunization with Bac_RSVM2 can effectively boost RSV M2-specific, IFN- γ -producing CD8⁺ T cells following RSV infection.

Protective efficacy in Bac_RSVM2-immune mice following RSV challenge

We next investigated whether RSV M2-specific CD8⁺ T-cell responses induced by Bac_RSVM2 confer protective immunity against RSV infection in mice. All immune mice were challenged with 10^6 PFU of RSV, and lung viral titers were determined by performing standard plaque assays with lung supernatants obtained from the immune mice at 5 days post-challenge (Fig. 4). In both IN and IM groups, Bac_

Fig. 4. Protective efficacy of Bac_RSVM2 following RSV challenge. All mice were challenged with 10^6 PFU of RSV A2 via intranasal route. At 5 days post-challenge, the lung viral titers were measured by standard plaque assay in HEP-2 cells. The limit of detection is 100 PFU/g of lung tissue. Data are presented as mean $\pm$ SD, and significant differences from the PBS are marked with asterisks. * $P < 0.05$.

RSVM2 immunization markedly suppressed RSV replication in the lungs compared with PBS and Bac_control groups. These results suggest that immunization with Bac_RSVM2 can provide protective immunity against RSV infection. Bac_control groups also showed slight reduction of lung viral titer at day 5 post-challenge, when compared with PBS groups ($P < 0.05$). We and other groups previously reported that

immunization with wild-type baculovirus provided partial protection against RSV (Kim and Chang, 2012) and influenza virus challenge (Abe *et al.*, 2003). Because baculovirus itself can induce innate immunity by stimulating Toll-like receptor (TLR) (Abe *et al.*, 2003, 2005, 2009; Chen *et al.*, 2009), this property might have conferred non-specific partial protection observed in Bac_control groups.

Fig. 5. Bac_RSVM2 does not cause severe body weight loss and pulmonary cell infiltration after RSV challenge. (A) At 28 days after immunization, all mice were challenged with 10^6 PFU/mouse of RSV A2, and their body weight was monitored daily for 5 days after post-challenge. (B–D) The BAL cells were collected at 5 days post-challenge, and then stained with anti-CD45 antibody, anti-Siglec-F antibody, anti-Gr-1 antibody, and anti-CD11c antibody. The stained cells were analyzed by flow cytometry. Fig. 5B–5D show the number (left panel) and percentage (right panel) of immune cells among CD45⁺-gated cells. All data are presented as mean $\pm$ SD. Significant differences are marked with asterisks. * $P < 0.05$, compared with PBS; ** $P < 0.05$, compared with Bac_RSVM2 IM.

Bac_RSVM2 vaccine does not promote vaccine-enhanced immunopathology following RSV challenge

In the development of RSV vaccines, it is essential to check for vaccine-induced immunopathology. To investigate whether effector CD8⁺ T cells induced by Bac_RSVM2 promote vaccine-enhanced immunopathology or not, we monitored body weight change daily for 5 days after RSV challenge. As shown in Fig. 5A, all mice recovered immediately after a slight initial weight loss (about 8% reduction in initial body weight for 1 day after challenge). Especially, at 2 days after RSV challenge, mouse body weight loss was significantly higher in the Bac_RSVM2 IM group (~10% reduction on average) than in the PBS (~6% reduction on average, $P = 0.05$) or the Bac_RSVM2 IN groups (~6% reduction on average, $**P < 0.05$). However, all groups did not show any clinical signs such as lack of grooming or decreased activity (data not shown) during the 5 days. These results suggest that the CD8⁺ T-cell responses induced by Bac_RSVM2 do not enhance severe vaccine-associated pathology measured by weight loss or clinical symptoms.

In relation to the vaccine-enhanced illnesses, pulmonary eosinophilia is considered to be one of the pathogenic factors in RSV vaccine studies. In our previous study, we demonstrated that Bac-RSV/G immunization did not cause pulmonary eosinophilia in mice (Kim and Chang, 2012). Thus, we next investigated the level of eosinophils induced by Bac_RSVM2 immunization. All immunized mice were challenged with RSV A2, and bronchoalveolar lavage (BAL) cells were harvested at 5 days post-challenge. We stained the BAL cells with antibodies to distinguish the various types of immune cells. Very low levels and numbers of eosinophils were detected in all immunized groups (Fig. 5B) and there was no significant difference among the groups. Neutrophils were a little higher in the Bac_RSVM2 IN group than PBS and Bac_control groups (Fig. 5C). The numbers and percentages of alveolar macrophages (AMs) were similar in all groups and no significant differences were observed among the groups (Fig. 5D). Taken together, our results show that Bac_RSVM2 immunization does not cause pulmonary eosinophilia or excessive infiltration of immune cells into the respiratory tract, while conferring protection from RSV infection without promoting vaccine-enhanced diseases.

Discussion

In the RSV vaccine development, since some RSV vaccine candidates such as formalin-inactivated RSV (FI-RSV) vaccine or vaccinia virus expressing RSV G glycoprotein (vvG) revealed problems related to vaccine-enhanced immunopathology, safety is one of the most important prerequisites for successful RSV vaccines. In this regard, the baculovirus vector platform used in this study has significant advantages in terms of safety because it is non-pathogenic and has no pre-existing vector immunity to humans. For example, influenza vaccine FluBlok® (protein form, Protein Sciences Corporation), among the approved vaccines based on baculovirus expression vector (van Oers *et al.*, 2015), has demonstrated high safety and immunogenicity in clinical trials (Cox and Hollister, 2009; Cox and Hashimoto, 2011), and

is currently on the market. As a vaccine vector, baculovirus surface could be used to display certain membrane antigens with gp64-based strategy (Makela and Oker-Blom, 2006; Lu *et al.*, 2012), or baculovirus genome could be engineered to have expression cassettes for mammalian expression of foreign antigens (Zhu *et al.*, 1998; Kost and Condreay, 2002). Previously, our group developed the safe and efficacious baculovirus-based RSV vaccine (Kim and Chang, 2012) and influenza virus vaccine (Sim *et al.*, 2016) using the baculovirus display technique. Here, in order to develop a CD8⁺ T-cell-inducing RSV vaccine, we constructed a recombinant baculovirus, Bac_RSVM2, by inserting the expression cassette containing RSV M2 gene located downstream of CMV immediate early promoter into the genome of baculovirus. Then, we immunized mice with Bac_RSVM2 through IN (mucosal immunization) and IM (systemic immunization) routes to prime the RSV M2-specific memory CD8⁺ T-cell responses by expressing the encoded RSV M2 protein inside the host cells. When exposed to RSV, these primed memory CD8⁺ T cells specific for RSV M2 might rapidly expand and form effector CD8⁺ T cells. We clearly showed that IN and IM immunization with Bac_RSVM2 effectively primed the M2-specific CD8⁺ T cells and boosted M2-specific IFN- γ -secreting effector CD8⁺ T cells in the lungs after RSV challenge, resulting in the suppression of RSV replication in the lungs without severe weight loss and pulmonary eosinophilia.

Interestingly, Bac_RSVM2 IM immunization tended to induce a higher level of RSV M2-specific CD8⁺ T cell population than Bac_RSVM2 IN immunization did. Furthermore, significant difference in the lung viral titers was observed between Bac_RSVM2 IN and IM immunization groups ($P < 0.01$), indicating that IM immunization controlled the virus replication more effectively than IN immunization did. We think that this might be because frequency of the RSV M2-specific CD8⁺ T-cell responses can differ depending on immunization route. In general, it is known that systemic administration elicits systemic immune responses more effectively than mucosal administration does (Holmgren and Czerkinsky, 2005; Su *et al.*, 2016). Moreover, an avidity and/or activity of functional CD8⁺ CTL was shown to be generally higher in systemic immunization compared to mucosal immunization (Belyakov and Ahlers, 2009). Our Bac_RSVM2 vaccine was designed to induce memory CD8⁺ T-cell responses specific for RSV M2, and indeed, IM immunization increased the frequency of specific CD8⁺ T cells more effectively than IN immunization upon RSV infection in the lungs. Supporting our results, several studies have reported that IM immunization induces more effector and effector memory CD8⁺ T cells than mucosal immunization (nasal or oral) does, which overall increased survival rate (Patel *et al.*, 2007; Malm *et al.*, 2015). Taken together, we assume that the central/effector memory CD8⁺ T cells primed by the Bac_RSVM2 IM immunization exerted better control of RSV replication in the lungs than the Bac_RSVM2 IN immunization.

New-born babies and the elderly are thought to be the primary target populations of RSV vaccine. The previous studies have shown that immune systems of newborns are immature, and the frequencies of RSV-specific CD8⁺ T cells were relatively low in infants compared with those in adults after RSV infection (Garcia *et al.*, 2000; Ruckwardt *et al.*, 2011).

Also, with respect to changes in the T-cell repertoire with aging, the elderly showed age-associated diminution in CD8⁺ T-cell responses (de Bree *et al.*, 2005; Nikolich-Zugich *et al.*, 2012; Fulton *et al.*, 2013). Therefore, Bac_RSVM2 vaccine may be suitable for the target populations with weak or diminished capacity for CD8⁺ T-cell responses since it could successfully prime the RSV-specific CD8⁺ T cells without vaccine-enhanced diseases upon RSV infection.

In summary, our study demonstrates that RSV M2-specific CD8⁺ T-cell responses induced by Bac_RSVM2 immunization via IN or IM route provide protective immunity against RSV infection without causing any vaccine-enhanced pathology or systemic diseases. Thus, our baculovirus-based RSV vaccine could be further developed as an efficacious and safe vaccine for future human use.

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