CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

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Vaccine containing G protein fragment and recombinant baculovirus expressing M2 protein induces protective immunity to respiratory syncytial virus

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Purpose: Respiratory syncytial virus (RSV) can cause serious respiratory illnesses such as pneumonia, asthma, and bronchiolitis in infants and elderly or immunocompromised individuals. An RSV vaccine has yet to be developed; only prophylactic anti-RSV antibody is commercially available. So, we investigated whether our vaccine candidate is able to induce type 1 CD4+ T helper (Th1), CD8+ T-cell responses, and protective immunity without vaccine-enhanced disease (VED) against RSV.

Materials and Methods: We used RSV G protein fragment (Gcf A) with recombinant baculovirus capable of expressing the RSV M2 protein (Bac M2) as a vaccine candidate, and injected this vaccine (Gcf A/Bac M2) intramuscularly, and challenged with RSV intranasally into mice. Enzyme-linked immunosorbent assay, flow cytometry, plaque assay, and weight measurement were performed to confirm humoral immunity, cellular immunity, and protective immunity.

Results: The Gcf A/Bac M2 formulation induced a stronger IgG response to Gcf A than Gcf A inoculation alone, and the ratio of IgG1/IgG2a indicated that the responses shifted predominantly to Th1. In addition, both RSV G-specific Th1 responses and RSV M2-specific CD8+ T-cell responses were induced, and G protein-associated eosinophilic infiltration was suppressed compared to the control group. Moreover, the Gcf A/Bac M2 group showed effective protection after an RSV challenge.

Conclusion: Bac M2 could serve as a vaccine with intrinsic adjuvant activity, and the Gcf A/Bac M2 shows promise as a vaccine candidate for inducing protective immunity without inciting VED.

Keywords: Respiratory syncytial virus, Vaccine, Recombinant baculovirus, M2, G protein

Introduction

Respiratory syncytial virus (RSV) is a *Pneumoviridae* family, negative-sense, single-stranded RNA virus that can cause respiratory diseases such as pneumonia, bronchiolitis, and asthma in infants and elderly or immunocompromised patients [1]. It is known that in the United States alone, more than 500,000 people visit the emergency room every year and more than 50,000 are hospitalized due to RSV. Worldwide, approximately 66,000-199,000 people die annually due to RSV infection, with most fatalities occurring in developing countries [2,3]. Since the discovery of RSV in 1956, only

the prophylactic antibodies palivizumab (Synagis) and RSV immunoglobulin (RSV-IVIG, RespiGam) have been commercially available, while no vaccine or medicine has been developed as yet [4,5]. In the 1960s, there were reports of deaths of children vaccinated with formalin-inactivated RSV (FI-RSV) vaccine due to vaccine-enhanced disease (VED), which is characterized by excessive eosinophil infiltration and type 2 CD4+ T helper (Th2)-like responses [6]. From this perspective, it is generally recognized that monitoring for elicited Th2-like and eosinophilic responses is important in the development of RSV vaccines.

The RSV G protein is a surface glycoprotein composed of 298 amino acids and is one of the main target proteins in RSV vaccine research. This protein is known to induce neutralizing antibodies, and to have a CX3C chemokine motif (a.a. 182-186) in the central conserved region capable of binding to CX3CR1, thereby influencing T-cell responses in RSV-infected lung [3,7]. Previously, a Gcf A of 131-230 amino acids from an RSV A2 strain was produced and evaluated as a vaccine with a cholera toxin (CT) adjuvant. As a result, specific IgG was induced, and protective efficacy against RSV A2 challenge was demonstrated [8,9]. However, because the CT cannot be used as an adjuvant in humans, our experiment was conducted using Bac M2, which has intrinsic adjuvant activity.

Baculovirus is a double stranded DNA insect virus, known to be a safe vaccine platform for human use because it cannot be replicated in mammalian cells. Several studies of the baculovirus platform have been conducted to investigate the expression of proteins in mammalian cells, using strong promoters including CMV, CAG, SV40, and HBV. Based on this research, Bac M2 was generated. The RSV M2 protein is expressed from two overlapping frames (ORFs) to M2-1 and M2-2, respectively. The M2-1 protein has a strong epitope for CD8+ T cells (a.a. 82-90), effectively inducing a cytotoxic T lymphocyte (CTL) response and contributing to virus clearance [10,11]. It has been shown that the M2-specific CTL responses are induced in BALB/c mice infected with vaccinia virus RSV-M2 (vac-M2) [12]. Previous studies have shown that M2-specific CTL responses are induced by Bac M2 immunization via intranasal or intramuscular routes [10,13].

In this study, we investigated whether Bac M2 can play an adjuvant role when used in combination with Gcf A by measuring humoral and cellular immune responses, bronchoalveolar lavage (BAL) cell responses, and viral titers in the lung as an indicator of protective immunity, as well as immunopathology-related weight loss.

Materials and Methods

Construction of a plasmid capable of expressing Gcf A and the production of Gcf A

The method for constructing a plasmid in which Gcf A was encrypted was as described in a previously published report [9]. Features of Gcf A include a central conserved region and cysteine residues (Cys-172, Cys-176, Cys-182, and Cys-186) [9]. The plasmid was transformed into ClearColi BL21 (DE3) (Lucigen, Middleton, WI, USA), then spread on a Luria-Bertani (LB) agar plate containing ampicillin and cultured overnight at 37°C. The single colony of Gcf A-transformed ClearColi was taken, added to fresh LB (+ampicillin) media (13 mL), and placed in a 37°C shaking incubator overnight. Fresh LB media (+ampicillin) (500 mL) was filled with 5 mL of ClearColi (+Gcf plasmid) that had been cultured the day before and grown up to 0.5 of OD 600 during shaking incubation. After the addition of 1 mM IPTG and shaking incubation at 37°C for 4 more hours, cells were obtained by centrifugation at 8,750 rpm and 4°C for 20 minutes. After cell resuspension using a binding buffer (20 mM KPO$_4$, 500 mM NaCl, 10 mM imidazole), the cells were disrupted with a sonicator. After adding a binding buffer to HisTrap HP (5 mL, GE Healthcare, Pittsburgh, PA, USA) containing Ni^{2+} Sepharose, the proteins that had not reacted to Ni^{2+} were removed. Elution buffer (20 mM KPO$_4$, 500 mM NaCl, 500 mM imidazole) was applied to obtain Gcf A reacting with Ni^{2+}. Gcf A treated with 4× sodium dodecyl sulfate (SDS) (+DTT) was run on 15% polyacrylamide gel electrophoresis (PAGE) gel after staining with coma blue reagent, confirming the presence of a band of Gcf A. Using Sephacryl S-200 (GE Healthcare), pure Gcf A was separated, and the concentration was measured using a BCA assay kit (Thermo Scientific, Waltham, MA, USA). Gcf A treated with 4× SDS (+DTT, -DTT) was run on 15% PAGE gel and was confirmed as pure Gcf A via banding after staining with coma blue reagent. The Gcf A was stored at -70°C.

Production of Bac M2

The method for production of a Bac M2 was as described in a previously published report [10]. Bac M2 was grown in *Spodoptera frugiperda 9* (Sf-9) insect cells (Invitrogen, Carlsbad, CA, USA) at 28°C using SF-900 II serum-free medium (Gibco BRL, Rockville, MD, USA). In order to obtain Bac M2, the final culture supernatants of infected Sf-9 cells were collected, treated with sucrose cushion (25% w/w sucrose in 5 mM NaCl, 10 mM EDTA in phosphate buffered saline [PBS]), and centri-

fuged at 24,000 rpm for 75 minutes at 4°C. The pellets were resuspended in 0.25 M sucrose and stored in LN_2. The viral titer was determined by plaque assay using Sf-9 cells according to the manufacturer's protocol (Invitrogen).

Production of the RSV A2 strain

To obtain RSV A2, HEp-2 cells (ATCC, Manassas, VA, USA) were cultured in minimal essential medium (MEM; Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS). RSV A2 was proliferated in MEM containing 3% heat-inactivated FBS, 2 mM glutamine, and 20 mM HEPES. After 4 days, syncytia formation was verified and the viruses were harvested by ultra-centrifugation (25,000 rpm for 1 hour at 4°C). The pellets were resuspended with a 25-gauge needle and serum-free MEM and then sonicated. Viral titers were performed by standard RSV plaque assay and the viruses were stored at -70°C.

Mouse immunization and RSV challenge

Six-week-old female BALB/cAnNCrljOri mice (n = 5/group) were purchased from Orient Bio Inc. (Seongnam, Korea) and kept in a specific pathogen free room for a week-long adaptation period. Three vaccines were used: Gcf A (20 μg/mouse) alone, Gcf A with added Bac control (live baculovirus; Gcf A/ Bac control, 1×10^7 plaque-forming unit [PFU]/mouse), and Bac M2 (baculovirus expressing M2 protein; Gcf A/Bac M2, 1×10^7 PFU/mouse) in Gcf A (20 μg/mouse). Immunization was performed on days 0 and 14 through intramuscular injection of the right leg (volume, 100 μL). Viral challenge was performed 23 days after the second immunization with RSV at a dose of 1×10^6 PFU/mouse. The mice were anesthetized with isoflurane (Ifran, Hana Pharm, Seongnam, Korea) inhalation and the RSV (volume, 50 μL) was administered via the left intranasal route. The condition of the mice was monitored through weight measurement for 5 days. The vaccines and RSV were diluted in PBS. All mice were monitored for the duration of the experiment and efforts were made to minimize pain. The research was carried out after approval by and in accordance with the guidelines of the Ewha Womans University Animal Experimental Ethics Committee (IACUC: 18-002).

IgG and IgG subtype titration specific to RSV G protein

Blood collection was performed two days before the first immunization and 21 days after the second immunization. Blood was drawn via the retro-orbital plexus using a capillary tube containing heparin and collected in an Eppendorf tube. The

blood was centrifuged for 15 minutes at 5,600 rpm and 4°C, and the sera were separated and stored at -70°C. The Gcf A-specific antibody titer was measured by direct enzyme-linked immunosorbent assay (ELISA). The Gcf A was diluted in PBS, held overnight in aliquots of 100 μL/well (50 ng/well) in a 96-well plate, washed 3 times with PBS (+0.05% Tween-20), and blocked with PBS (+0.05% Tween-20) containing 1% skim milk for 2 hours at room temperature (RT). After 2 additional washes with PBS (+0.05% Tween-20), 2-fold serial diluted serum was added to the coating antigen and incubated for 2 hours at RT. After 4 washes with PBS (+0.05% Tween-20), horseradish peroxidase-conjugated affinity-purified rabbit anti-mouse total IgG secondary antibody (Zymed Laboratory, San Francisco, CA, USA) was applied. For the second immunization serum, rabbit anti-mouse IgG1, IgG2a secondary antibody, horseradish peroxidase (Invitrogen) was applied. Incubation at RT for 1 hour was followed by washing 6 times with PBS. After the addition of tetramethylbenzidine peroxidase substrate (1:1; TMB, KPL, Gaithersburg, MD, USA), the reaction was stopped with 1 M H_3PO_4, and the analysis was carried out at OD 450 nm using a Thermo ELISA plate reader. Cut-off values were calculated with serum-free wells.

Sampling for investigation of cellular responses

On day 5 after the RSV challenge, the mice were sacrificed by CO_2 anesthesia. BAL fluids were collected with a catheter (BD Angiocath Plus, Becton-Dickinson, Franklin Lakes, NJ, USA) and 1 mL of PBS. Cells and supernatants were separated by centrifugation and leukocyte recruitment was measured in the BAL cell samples. Lung tissues of the immunized mice were collected, homogenized using a 70 μm cell strainer (SPL), and centrifuged to separate lung mononuclear cells and supernatants. Then, the proportions of immune cells were measured and the supernatants were used to measure viral replication in the lungs.

### Lung viral titration specific for the RSV A2 strain

On day 5 after the RSV challenge, all mice were sacrificed by CO_2 euthanasia and single-cell suspensions of lung tissue were obtained using MEM (Welgene) and a 70 μm cell strainer (SPL). The cells and supernatants were separated by centrifugation at 1,600 rpm and 4°C and the supernatants were incubated at 37°C for 5 days with HEp-2 cells (ATCC). Viral titers were recorded as PFU/g of lung tissue, with a limit of detection of 100 PFU/g.

Flow cytometric analysis

To measure the granulocyte population in the BAL specimens, the BAL cells were treated with rat anti-mouse CD16/CD32 blocking antibody (BD Biosciences, San Jose, CA, USA) and reacted at RT for 10 minutes. BAL cells were stained in the dark for 30 minutes at 4°C with anti-Siglec-F antibody (E50-2440, BD Biosciences), anti-Gr-1 antibody (RB6-8C5, BioLegend, San Diego, CA, USA), anti-CD11c antibody (N418, BioLegend), and anti-CD45 antibody (30-F11, BioLegend). To analyze the RSV M2-tetramer binding activity of CD8$^+$ T cells, lymphocytes isolated from lung tissue were treated with CD16/32 blocking antibody (BD Biosciences) and reacted at RT for 10 minutes. Staining was performed at 4°C for 30 minutes in the dark using with anti-CD8 antibody (53-6.7, BioLegend), anti-CD44 antibody (IM7, BioLegend), and MHC class I H-2K^d tetramer complexes (H-2K^d/SYIGSINNI) presented with RSV M2$_{82-90}$ peptide. All stained cells were fixed with fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences) and analysis was performed with a BD FACSCalibur (BD Biosciences).

Th1 interferon γ (IFN-γ)$^+$ cells and CD8$^+$ IFN-γ$^+$ T cells were analyzed by intracellular staining (ICS). First, lung lympho-cytes were resuspended in IMDM (Welgene) containing 10% heat-inactivated FBS. The cells were stimulated by adding 50 μg/mL phorbol myristate acetate (1:1,000, Sigma-Aldrich, St. Louis, MO, USA), 500 μg/mL ionomycin (1:1,000, Sigma-Aldrich), and either 10 μM RSV M2 peptide (82-90: SYIGSINNI) or 10 μM RSV G peptide (183-195: WAICKRIPNKKPG) to IMDM (+10% FBS) (Welgene) containing β-mercaptoethanol (1:1,000, Sigma-Aldrich), recombinant human IL-2 (BioLegend), and Brefeldin A (1:1,000, Invitrogen), followed by incubation in the dark for 5 hours at 37°C. Stimulated cells were stained with anti-CD3 antibody (17A2, BioLegend), anti-CD44 antibody (IM7, BioLegend), and either anti-CD8 antibody (53-6.7, BioLegend) or anti-CD4 antibody (RM 4-5, BioLegend) at 4°C for 30 minutes in the dark and fixed with FACS lysing solution (BD Biosciences). For the intracellular cytokine staining, first the fixed cells were treated with FACS buffer (0.5% FBS, 0.09% NaN$_3$ in PBS) containing 0.5% saponin (Sigma-Aldrich) and then they were permeabilized at RT for 15 minutes in the dark. The permeabilized cells were stained with anti-IFN-γ antibody (XMG 1.2, BioLegend) at RT for 30 minutes in the dark. The stained cells were analyzed using a BD FACSCalibur (BD Biosciences) and all flow cytometry da-
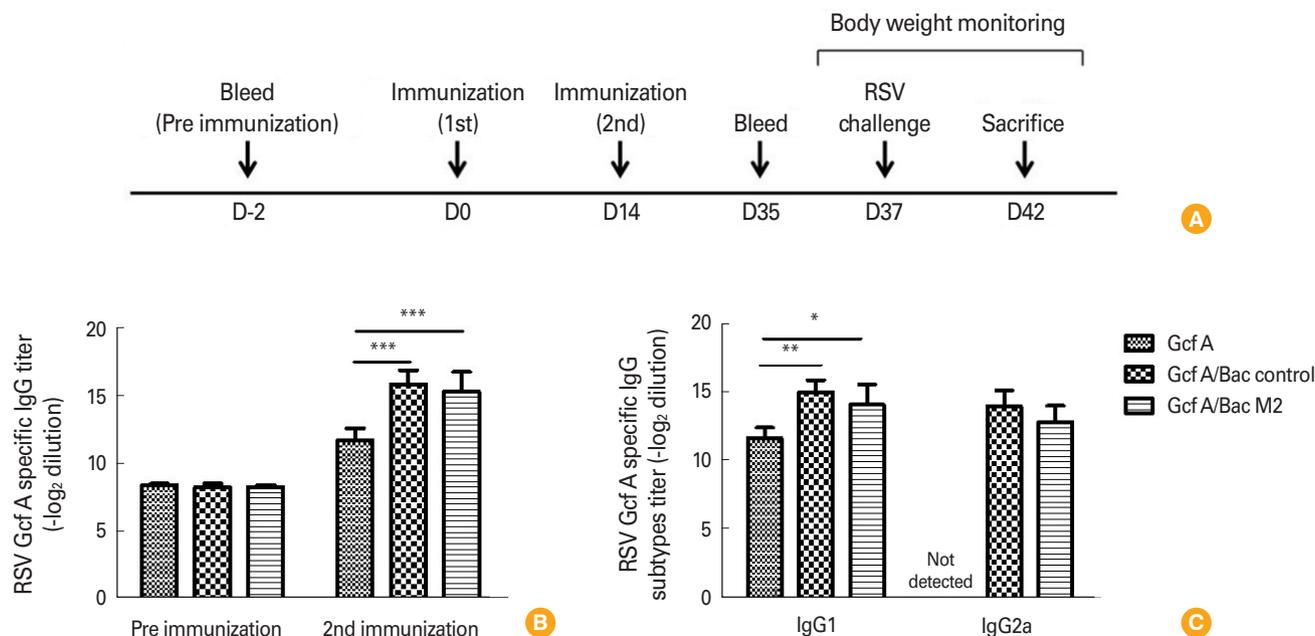
Fig. 1. Experiment schedule and assessment of the vaccine-induced humoral immune responses. (A) Experiment schedule for investigating the efficacy of the manufactured vaccine. (B) Serum IgG antibody titers specific for Gcf A were measured by enzyme-linked immunosorbent assay (ELISA) using sera obtained two days before and at days 0 and 14 after immunization with Gcf A, Gcf A/Bac control, and Gcf A/Bac M2 (20 ug Gcf A and 10^7 plaque-forming unit Bac control and Bac M2 were used). (C) IgG subtypes (IgG1, IgG2a) specific for Gcf A were measured by ELISA using serum IgG antibodies obtained after the second immunization. RSV, respiratory syncytial virus. Results indicate Log$_2$ endpoint values from individual mice (n=5/group). Statistically significant values are marked with an asterisk. *p<0.05, **p<0.01, ***p<0.001.

ta were analyzed using Flowjo software (TreeStar Inc., San Carlos, CA, USA).

Statistical analysis

ANOVA and Bonferroni multiple comparisons test were used to determine the statistical difference between each group. The p-value was considered to be less than 0.05 when there was more than 95% confidence interval. Statistically significant values are marked with an asterisk.

Results

Experiment scheme and vaccine-induced antibody responses specific to Gcf A

We conducted the study according to the experimental plan shown in Fig. 1A and investigated the immunogenicity of each vaccine by comparing the Gcf A-specific IgG titer in each group through ELISA using sera obtained before immunization and after two immunizations (Fig. 1A, B). We found that the mean titers of serum antibodies increased considerably after the second immunization, and confirmed that the Gcf A/Bac control and Gcf A/Bac M2 groups had higher titers than the Gcf A group (Fig. 1B). After the failure of the FI-RSV vaccine, it has generally been thought that the Th2 response must be avoided and inducing the Th1 response is important for preventing VED [14]. Therefore, before verifying the responses of Th1 IFN-γ⁺ cells, we compared the IgG subtype titers that can indicate the responses of Th1 or Th2 cells using the sera from each group. Doing so, we confirmed that the Gcf A/Bac con-

trol and Gcf A/Bac M2 groups (reciprocal log2 titers, 14 and 15, respectively) showed higher mean titers of serum IgG1 antibodies than the Gcf A group (reciprocal log2 titer, 11). Importantly, no IgG2a response was observed in the Gcf A group, while it was evident in the other two groups (reciprocal log2 titers, 14 and 13, respectively). These results indicated that total IgG response was upregulated by Bac control or Bac M2 when co-immunized with Gcf A, and importantly, significant IgG2a responses were induced only in Bac co-immunization groups (Fig. 1C).

Th1 IFN-γ⁺ cell responses induced by vaccination

IL-2 secretion from Th1 cells activates T cells and induces antibody production by promoting B cell maturation. In addition, Th1 cells secrete IFN-γ, which can be helpful for increasing CTL activity and suppressing virus replication [14-16]. In order to identify the Th1 INF-γ⁺ cells specific for RSV G when the Bac M2 was used as an adjuvant, we prepared lung cells of immunized mice on day 5 after the RSV challenge, stimulated them with RSV G peptide (183-195: WAICKRIPNKKPG), and enumerated the percentages of Th1 cells secreting IFN-γ. Th1 INF-γ⁺ cells were significantly more strongly induced in the Gcf A/Bac M2 group (mean Th1 cells, 4.5%) compared with the Gcf A group (Fig. 2). Interestingly, the Gcf A/Bac control group also showed a significant Th1 INF-γ⁺ cell response compared with the response in the Gcf A group (Fig. 2B). Even, the percentage of cell population was slightly higher in comparison with the Gcf A/Bac M2 group, though the difference was not statistically significant (Fig. 2).
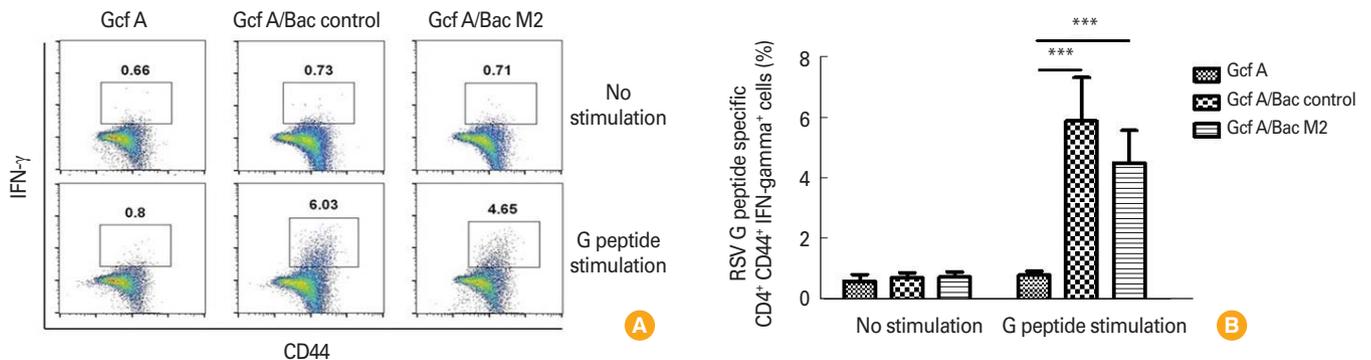
Fig. 2. Identification of CD4⁺ Th1 interferon γ (IFN-γ)⁺ cells specific for RSV G (183-195) peptide induced by vaccine inoculation. Lung mononuclear cells were isolated from mice vaccinated twice and sacrificed 5 days after RSV challenge. Isolated cells were stimulated with G peptide (183-195: WAICKRIPNKKPG) for 5 hours *in vitro*. Cells were stained with anti-CD3 antibody, anti-CD4 antibody, and anti-CD44 antibody and then fixed. Permeabilized cells were stained with anti-IFN-γ antibody and analyzed by flow cytometry. (A) The dot plots in the gated area represent CD4⁺ CD44⁺ T cells (Th1 cells) that can secrete IFN-γ. (B) The percentage of CD4⁺ CD44⁺ T cells (Th1 cells) that can secrete IFN-γ for each vaccine-immunized group. Data are expressed as mean± standard deviation (n=5/group). Statistically significant values are marked with an asterisk. ***p<0.001.

## CD8+ IFN-γ+ T-cell responses specific for RSV M2 induced by vaccination

IFN-γ secreted by CD8+ T cells during RSV infection is important because it can block the pulmonary eosinophils; this has been observed in experiments with mice immunized with a recombinant vaccinia virus (vacv M2) capable of expressing M2 protein [17]. In order to investigate the CTL response specific to RSV M2, we confirmed the percentages of CD8+ IFN-γ+ T cells by stimulation with M2 peptide (82-90: SYIG-SINNI) in lung cells obtained on day 5 after the RSV challenge. When Gcf A-Gcf A/Bac M2 and Gcf A/Bac control-Gcf A/Bac M2 groups were compared, the response of CD8+ IFN-γ+ T cells specific to M2 peptide was shown to be significantly much

higher in the Gcf A/Bac M2 group (~6.2% vs. ~22.0%) (Fig. 3). There was no significant difference in the responses of CD8+ IFN-γ+ T cells between the Gcf A group (mean CD8+ T cells, 6.5%) and Gcf A/Bac control group (mean CD8+ T cells, 5.7%).

Identification of CD8+ M2 Tet+ T cells after RSV challenge

Following identification of CD8+ IFN-γ+ T-cell responses, lung cells obtained 5 days after RSV challenge were also checked to confirm whether they are also reactive to M2 peptide-loaded MHC class I H-2K^d tetramer (H-2K^d/SYIGSINNI). The percentage of CD8+ Tet+ cells in the Gcf A/Bac M2 group (~58.8%) appeared to be about 2- to 3-fold higher than in the other two groups, while the percentages of these cells in the Gcf A group
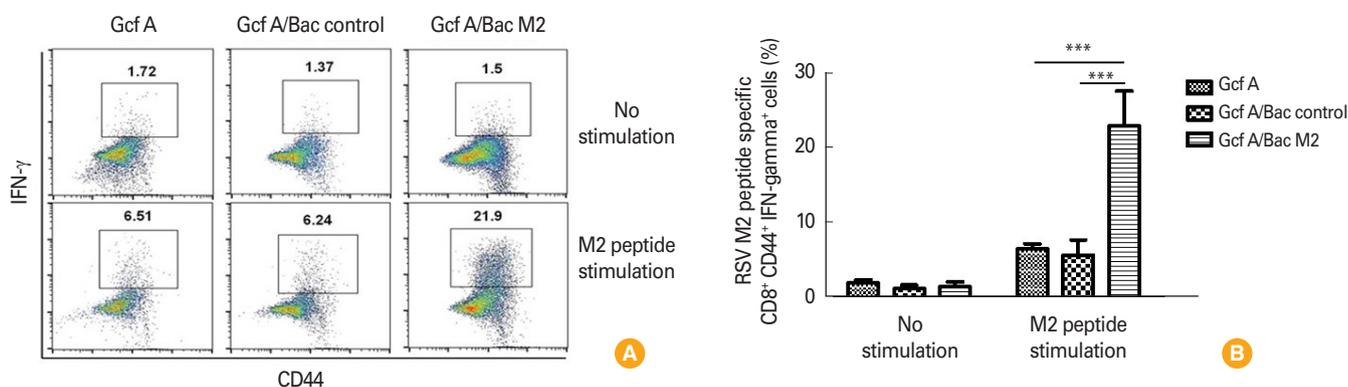
Fig. 3. Comparison of CD8+ interferon γ (IFN-γ)+ T cells specific for RSV M2 (82-90) peptide after vaccination. Mice were challenged with RSV after 2 vaccinations and lung mononuclear cells were harvested at day 5 to identify CD8+ CD44+ T cells capable of secreting IFN-γ. The harvested lung mononuclear cells were stimulated with M2 peptide (82-90: SYIGSINNI) for 5 hours *in vitro*. Stimulated lung cells were stained with anti-CD3, anti-CD8, and anti-CD44 antibodies and then fixed. Permeabilized cells were stained with anti-IFN-γ antibody and analyzed by flow cytometry. (A) The dot plots in the gated area represent the CD8+ CD44+ T cells that can secrete IFN-γ. (B) The percentage of CD8+ CD44+ T cells that can secrete IFN-γ for each vaccine-immunized group. Data are expressed as mean± standard deviation (n=5/group). Statistically significant values are marked with an asterisk. ***p<0.001.
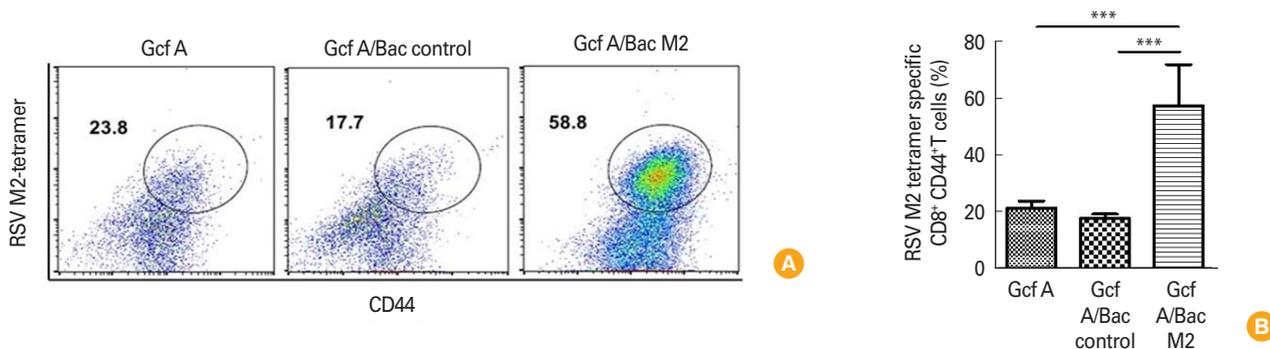

Fig. 4. CD8+ Tet+ cells specific for RSV M2 after vaccination. To determine the binding capacity between CD8+ T cells and the RSV M2 tetramer, lung mononuclear cells were isolated from mice sacrificed at day 5 after RSV challenge. Isolated cells were stained with anti-CD8 antibody, anti-CD44 antibody, and H-2K^d RSV M2 tetramer and then fixed. Fixed cells were analyzed by flow cytometry. (A) Gating represents the population of CD8+ CD44+ T cells that can bind to the RSV M2 tetramer. (B) The percentage of CD8+ CD44+ T cells specific for the RSV M2 tetramer is shown for each group. Data are expressed as mean±standard deviation (n=5/group). Statistically significant values are marked with asterisks. ***p<0.001.

(~23.8%) and Gcf A/Bac control group (~17.7%) in the same gated cell population were similar (Fig. 4A). The similar percentages of CD8⁺ Tet⁺ cells in the Gcf A and Gcf A/Bac control groups and the significantly higher mean percentage of CD8⁺ Tet⁺ cells in the Gcf A/Bac M2 group were confirmed (Fig. 4B).

Identification of VED by measurement of BAL cells

The detection of VED in has been integral to RSV vaccine development since the failure of the FI-RSV vaccine, which was found to cause lung inflammation due to hypersecretion of type II cytokines. Therefore, our study included investigation of the extent of eosinophil, alveolar macrophage, and neutrophil response for each group in the BAL cells collected from the airway. First, we found that the numbers of eosinophils (CD11c⁻, Siglec-F⁺) in Gcf A and Gcf A/Bac M2 groups appeared to be lower, whereas the Gcf A/Bac control group had a slightly greater number (Fig. 5A, C). Next, we observed that the alveolar macrophage (CD11c⁺, Siglec-F⁺) responses followed
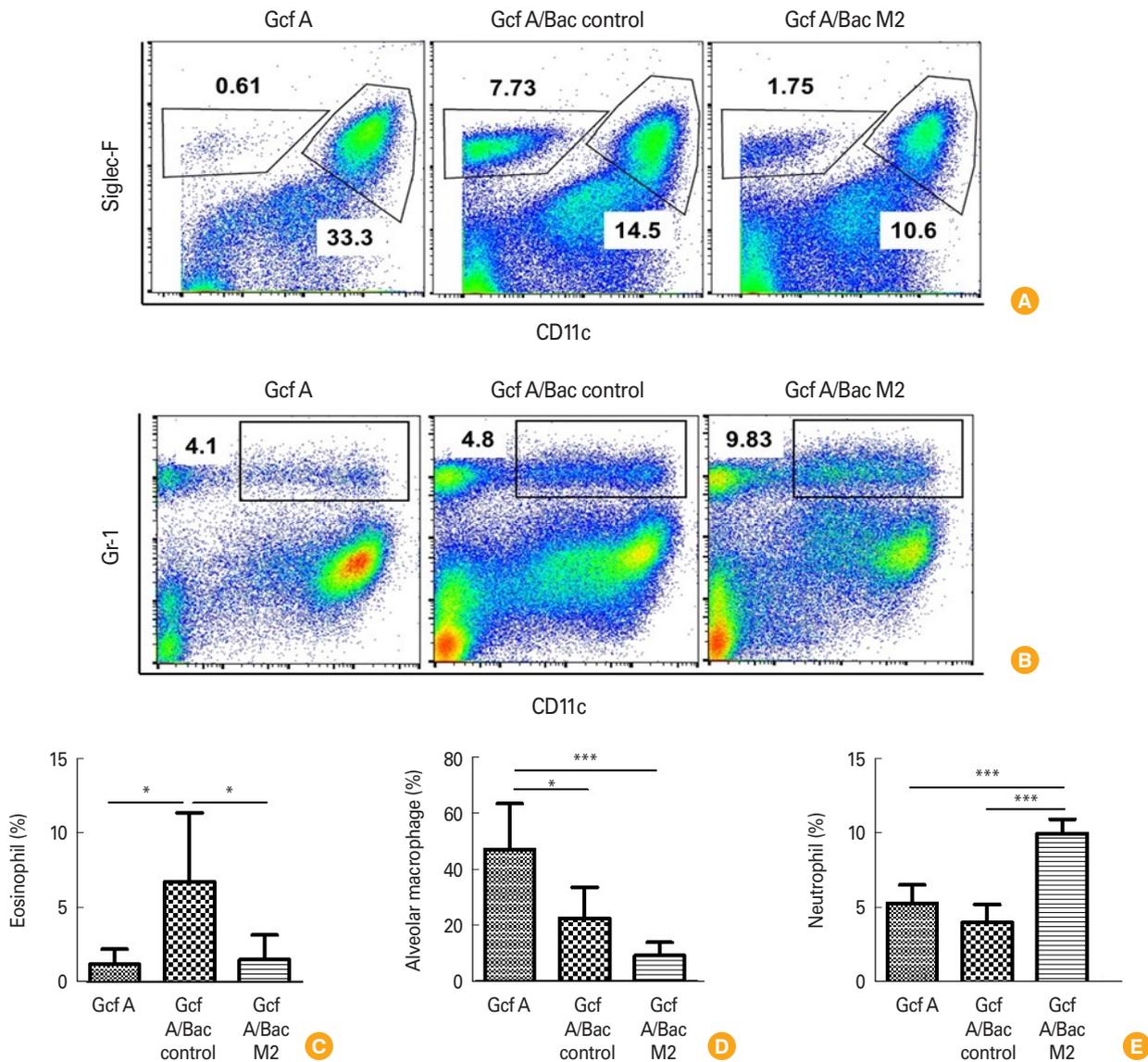
Fig. 5. Determination of vaccine-enhanced disease through analysis of bronchoalveolar lavage (BAL) cells. After 5 days of RSV challenge, BAL cells were harvested from the airways of all immunized mice. BAL cells were stained with an anti-CD45 antibody, anti-CD11c antibody, anti-Siglec-F antibody, and anti-Gr-1 antibody. The stained cells were fixed and analyzed by flow cytometry. (A) CD11c⁻, Siglec-F⁺ cells are eosinophils and CD11c⁺, Siglec-F⁺ cells are alveolar macrophages. (B) CD11c⁺, Gr-1⁺ cells are neutrophils. (C-E) Percentage of BAL cells for each group: eosinophils (C), alveolar macrophages (D), and neutrophils (E). Data are expressed as mean±standard deviation (n=5/group). Statistically significant values are marked with an asterisk. *p<0.05, ***p<0.001.
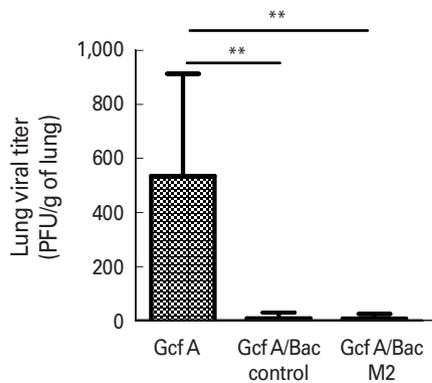
Fig. 6. Vaccine-induced protective immunity against RSV A2 challenge. Five days after the RSV A2 strain challenge (1×10^6 plaque-forming unit), lung supernatants harvested from all mice in each group were reacted with HEp-2 cells and incubated for 5 days. Lung viral titers were measured by standard plaque assay in HEp-2 cells, and the limit of detection was set at 100 PFU/g of the lung. Data are expressed as mean±standard deviation (n=5/group). Statistically significant values are marked with an asterisk. **p<0.01.

Fig. 7. Body weight change after RSV challenge. Immunization was performed on days 0 and 14, and RSV challenge was performed 23 days later. Body weight of all mice was measured for 5 days as a marker of immunopathology. Data are expressed as mean±standard deviation (n=5/group). Statistically significant values are marked with an asterisk. *p<0.05, **p<0.01.
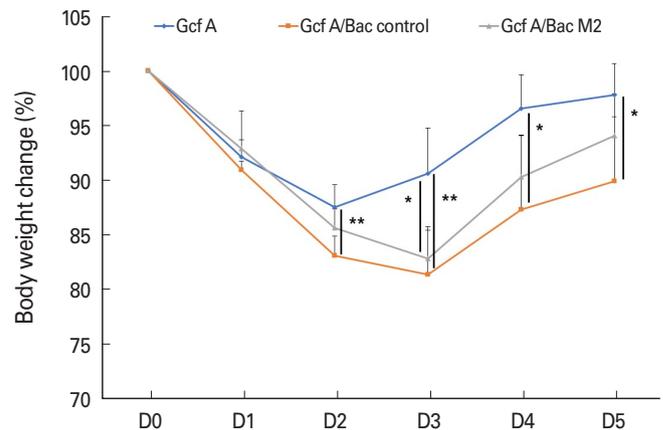
the order Gcf A group>Gcf A/Bac control group>Gcf A/Bac M2 group. Statistical significance was confirmed for the Gcf A/Bac control and Gcf A/Bac M2 groups compared with the Gcf A group, and for the Gcf A/Bac M2 group compared with the Gcf A/Bac controls (Fig. 5A, D). Finally, we found that the neutrophil (CD11c$^+$, Gr-1$^+$) response was highest in the Gcf A/Bac M2 group compared to a level of approximately 4%-5% in the Gcf A and Gcf A/Bac control groups (Fig. 5B, E).

Vaccination-induced protective immunity against an RSV A2 challenge

Immunized mice were challenged with RSV to determine if the immune responses produced by the vaccination were protective against virus infection. Five days after the infection challenge, we performed plaque assays on the lung supernatants. The Gcf A group was verified to have 537 PFU/g of the lung, while the Gcf A/Bac control and Gcf A/Bac M2 groups showed no sign of virus replication (results below the limit of detection), indicating that these vaccines provide protection against RSV infection (Fig. 6).

Observation of immunopathology through body weight change during RSV challenge

Body weight monitoring is important because the excessive inflammatory responses in the lung during RSV infection are directly linked to weight loss. So, weight loss was checked for 5 days after the RSV challenge as a marker of immunopathol-

ogy. The Gcf A group showed decreased body weight until day 2 but body weight increased to 98% of baseline on day 5. In contrast, Gcf A/Bac control and Gcf A/Bac M2 groups showed decreased body weight until day 3 and then body weight recovered. However, on day 3, body weight appeared to be 81% in the Gcf A/Bac control group and 83% in the Gcf A/Bac M2 group, which differed significantly compared with the body weights of the Gcf A group (Fig. 7). Furthermore, clinical disease scores were also low for the Gcf A/Bac control and Gcf A/Bac M2 groups on days 2 to 3, but the mice later recovered from these symptoms (data not shown).

Discussion

After the failure of the FI-RSV vaccine, the need for a vaccine that can avoid excessive type II cytokine responses has been emphasized [18,19]. For this reason, the development of vaccine candidates inducing neutralizing antibodies without biased T-cell responses and/or balanced Th1/Th2 cells is ongoing [16,20-22]. G protein is thought to be a suitable target protein for vaccine development because it can induce long-term neutralizing antibodies [15]. The baculovirus used in our study has self-adjuvanticity to activate the innate immune system and does not cause any of the significant side effects seen with lipopolysaccharide-like pathogen-associated molecular pattern. In addition, if appropriate enhancers or promoters are used in the baculovirus expression constructs, it is possible to

express antigens in transduced mammalian cells [10,23,24]. Following this concept, we combined the Gcf A (a.a. 131-230) containing the CX3C motif (a.a. 182-186) as an antigen and Bac M2, which expresses the M2 protein, as an adjuvant and evaluated the combination as a novel RSV vaccine candidate. We also examined cellular immune responses to determine whether Bac M2 could prime a specific T-cell response through the expression of M2 antigen in addition to adjuvant function. We investigated the presence of VED by examining the Th1 cell, CD8+ T cell, and BAL cell responses and performing body weight measurements. Finally, in order to test the effectiveness of the protective immunity against RSV induced by the vaccine, we performed lung virus titration analysis.

First, we compared the antibody responses of the three study groups. The Gcf A/Bac control and Gcf A/Bac M2 groups showed higher IgG, IgG1 (Th2), and IgG2a (Th1) responses than the Gcf A alone inoculation group. Interestingly, the IgG2a response was not observed in the Gcf A single inoculation group but was predominant in the other two groups, and the pattern of this result was consistent with the overall humoral immune response as presented in Fig. 2. These results are consistent with the results of several studies that demonstrated that vaccine substances containing baculovirus activate the nuclear factor κB, IRF-dependent pathway or the TLR9-MyD88 dependent pathway leading to IgG2a and Th1 type immune responses [25-29]. Inoculation of Gcf A alone also induced the humoral immune response and suppressed viral replication. This is consistent with the results of a previous study [9]. In addition, although Gcf A contained a strong I-E^d-restricted Th epitope within the antigen, inoculation of Gcf A alone did not induce VED. This result is also consistent with a previous study suggesting that Gcf A inoculation does not cause VED [9].

CTL responses were evaluated by tetramer binding activity and ICS analysis. The Gcf A/Bac M2 group showed a significantly higher response than the Gcf A and Gcf A/Bac control groups. This suggests that the M2 antigen is successfully expressed by Bac M2, which leads to M2-specific CTL priming and subsequent recruitment after RSV infection [30]. It is likely that M2-specific CTL responses primed by Bac M2 play an important role in virus clearance [31,32].

Next, we investigated VED-associated BAL cell responses. In the eosinophil analysis, the Gcf A/Bac control group showed the highest response. However, compared with the FI-RSV (positive control) group of our previous study, the Gcf A/Bac control does not seem to cause an excessive eosinophilic re-

sponse [9]. Also, the Gcf A/Bac M2 group's neutrophil responses were significantly higher than those of the other two groups. These results suggest that CTLs increase the penetration of neutrophils [33], consistent with the results shown in Figs. 3 and 4. On body weight measurement as another indicator of VED, we observed body weight reduction in Gcf A/Bac control and Gcf A/Bac M2 groups on the third day of the RSV challenge. Although the exact cause of the body weight reduction cannot be identified, the use of the adjuvant to increase the immune responses or the induction-boosting effect of the 2-dose vaccine inoculation seems to induce temporary excess immune responses resulting in weight loss. Nevertheless, the body weight began to recover on day 4, and the body weight returned to 90% in all groups on day 5 and the health status of the mice recovered to normal.

When protective immunity against the RSV A2 challenge was analyzed by lung viral titers, no virus was detected in the Gcf A/Bac control or the Gcf A/Bac M2 group compared to the Gcf A alone group. This might result from increased antibody-mediated virus neutralizing activity via the adjuvant effect of the baculovirus itself, as this pattern has already been demonstrated in other studies using baculovirus as an adjuvant [29].

Taken together, these results indicate that the Gcf A/Bac M2 vaccine composition induced humoral and cellular immune responses and provided protective immunity against RSV infection without VED. These results suggest that baculovirus may be a good platform for both adjuvant and antigen delivery in vaccine development. However, for application in humans, baculovirus should be further studied as a vaccine material, and appropriate concentration range and safety tests should be carefully conducted [29]. For example, further studies are needed to determine whether the reduction in body weight on days 2 and 3 after the RSV challenge was caused by baculovirus. Moreover, since baculovirus (Bac control) had effects similar to those seen in the Gcf A/Bac M2 group (except for the CD8+ T cell and BAL cell responses), it is worth verifying through further study whether the baculovirus itself can be used as an adjuvant.

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