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Brief communication

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Single-dose intranasal adenovirus-based RSV vaccines targeting G and M2

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We have developed adenovector-based vaccines targeting G and M2 protein of respiratory syncytial virus. Single intranasal vaccination induced robust G protein-specific IgG and IgA responses, as well as M2-specific $CD8^+$ T-cell immunity, and provided protection against RSV A and B infection. These findings suggest that this vaccine combination holds promising potential for effective RSV prevention.

Respiratory syncytial virus (RSV) is a significant contributor to illness and death globally, particularly affecting infants during their first 6 months of life, as well as the elderly and immunocompromised[1]. Despite the urgent need for an RSV vaccine, no approved vaccine has been available for over 60 years following the failure of the formalin-inactivated vaccine introduced in the 1960s[2]. Recently, the U.S. Food and Drug Administration (FDA) has approved two RSV vaccines, GSK AREXVY and Pfizer ABRYSVO, which are administered via intramuscular injection[3]. While most existing vaccines are administered via intramuscular injection, needle-free intranasal vaccines offer several advantages, such as being painless, easy to administrate, and capable of inducing a local immune response at the initial point of virus entry[4]. Therefore, the development of an intranasal RSV vaccine is critical to improve vaccine accessibility and efficacy.

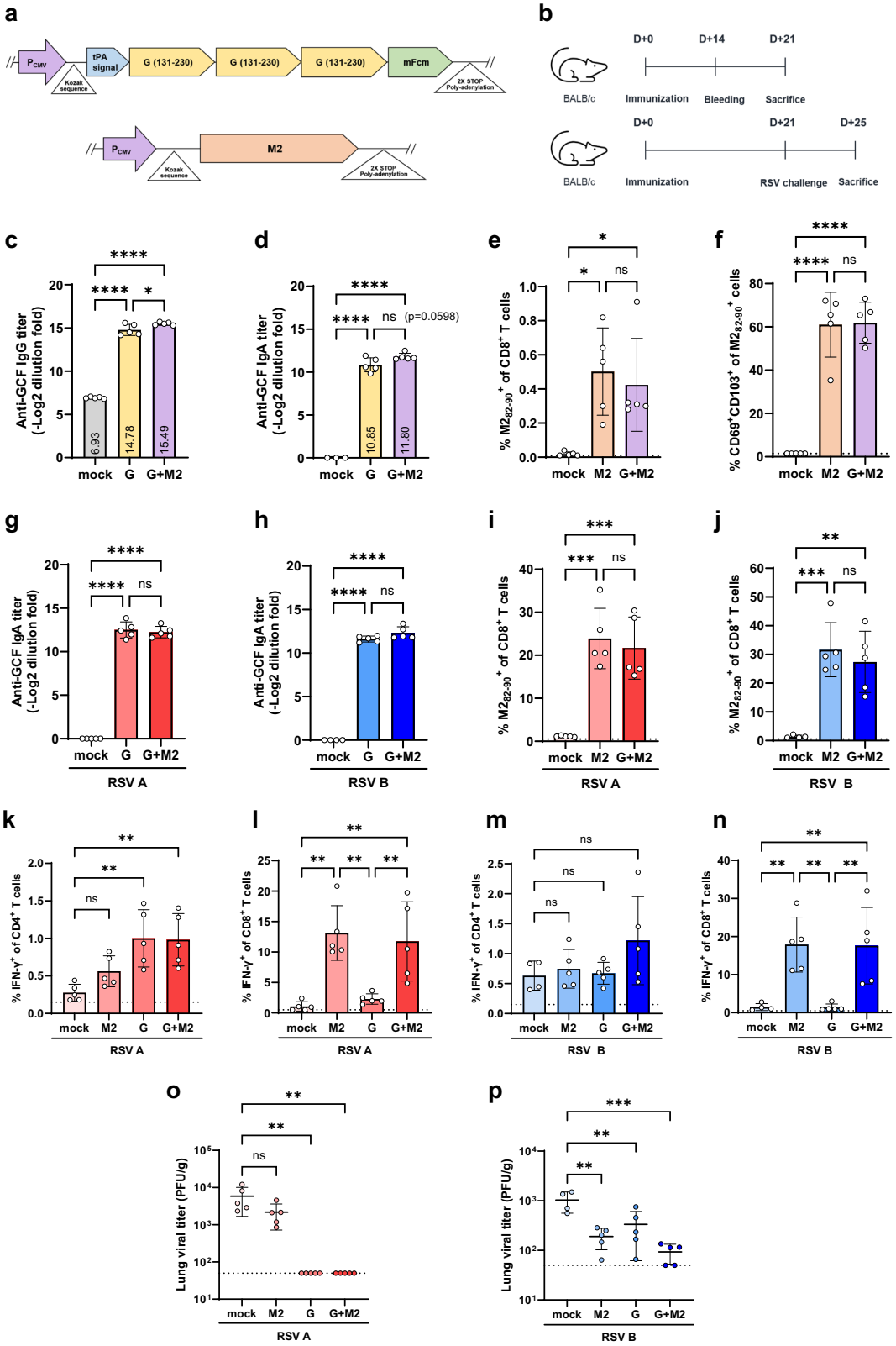
To develop a vaccine for intranasal administration, we selected an adenoviral vector platform because of its numerous advantages. Adenoviruses, which infect the mucosal surfaces of the respiratory tract, are highly effective in inducing mucosal immunity[5]. Recombinant adenoviruses (rAd), which are engineered by deleting the E1 gene to inhibit viral replication and the E3 gene to impair immune evasion, exhibit improved safety and immunogenicity, and are capable of inducing both innate and adaptive immune responses[5,6]. Furthermore, this platform is highly versatile, can easily insert foreign genes, and can be used for mass production[5]. Therefore, adenoviral vector platforms are promising candidates for future vaccines against infectious diseases.

RSV has a few proteins on its surface: attachment glycoproteins (G) for attachment to the host cell, fusion glycoproteins (F) that promote viral entry into the host cell by facilitating membrane fusion, and small hydrophobic proteins, which are ion channels[7,8]. Inside the virus, there are several essential components for viral replication, including nucleoproteins, phosphoproteins, M2 proteins, and polymerases[7,8]. The two major subtypes, RSV A and RSV B, are primarily differentiated by their G-protein sequences[9]. Among these viral proteins, we have developed vaccines targeting the G and M2 proteins. Only G and F proteins can induce neutralizing antibodies, and G protein contains a chemokine motif that functions as an immunomodulator, making it a promising candidate for vaccine targets[8]. However, G proteins are more variable between viral strains than F proteins[10]. Therefore, a vaccine targeting the G protein derived from RSV A sequence may be less protective against RSV B. To resolve this issue, we have developed a vaccine targeting the M2 protein, which is well conserved between different strains[10]. In addition, G protein elicits humoral immunity, whereas M2 protein triggers cell-mediated immunity, inducing a balanced immune response. Therefore, in this study, we developed adenovirus-based vaccines against G protein and M2 protein and evaluated the efficacy of the vaccine by intranasal administration.

The G protein-targeted vaccine comprises three G glycoprotein core fragments (GCFs) spanning the 131–230 amino acid (a.a.) positions, representing the most conserved region of the G protein sequence derived from the RSV A2 strain. These fragments are fused to a murine Fc receptor mutant (mFcm), a modified mouse-derived Fc receptor sequence engineered to enhance the stability and persistence of the vaccine antigen by preventing its clearance by phagocytes. Additionally, to further augment the vaccine's effectiveness, a tissue plasminogen activator (tPA) signal sequence was added upstream of the GCF sequence to promote efficient secretion of the antigen from the cell (Fig. 1a). Western blot analysis confirmed the successful expression and secretion of the vaccine-encoded protein (Supplementary Fig. 1). For the M2-targeting vaccine, the open reading frame (ORF) of the M2-1 protein from the RSV A2 strain was inserted into a recombinant adenoviral vector (Fig. 1a). To determine the optimal combination ratio of the two vaccines, rAd-3xGmFcm and rAd-M2 were combined at ratios of 3:1 and 3:3. Comparative analysis revealed no statistically significant enhancement in immunogenicity and protective efficacy with increased M2 content, indicating that a 3:1 ratio was sufficient. This ratio was adopted for subsequent experiments (Supplementary Fig. 2). Mice were divided into four groups: rAd-mock (mock), rAd-M2 alone (M2), rAd-3 x GmFcm alone (G), and a 3:1 mixture of rAd-3xGmFcm and rAd-M2 (G + M2). Two parallel experimental sets were conducted. In one set, mice were vaccinated, and serum was collected at 2 weeks post-vaccination, followed by bronchoalveolar lavage fluid (BALF) and lung tissue collection at week 3 to assess immunogenicity. In the other set, mice were challenged with RSV at 3 weeks post-vaccination, and BALF and lung samples were collected 4 days post-infection to evaluate protective efficacy (Fig. 1b). To assess the humoral immune responses elicited by rAd-3xGmFcm, ELISA assays

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were performed on serum and BALF samples. GCF-specific IgG titers in serum were robust in both the G and G + M2 groups, with the G + M2 group exhibiting a statistically significant ~1.6-fold higher titer, suggesting a potential immune-enhancing effect of the M2 component (Fig. 1c). Similarly, IgA titers were elevated in both groups, with a ~1.9-fold increase observed in the G + M2 group compared to G alone, although this difference did not reach statistical significance (Fig. 1d). To evaluate cellular immunity, M2-specific $CD8^+$ T cells were quantified by $K^d/M2_{82-90}$ tetramer staining. Both M2 and G + M2 groups showed similar frequencies of antigen-specific $CD8^+$ T cells in the lungs (Fig. 1e). Analysis of tissue-resident memory T cells (TRM) also revealed high proportions in both groups, indicating the successful induction of localized memory responses

Fig. 1 | Comparative evaluation of the immunogenicity and protective efficacy of single and combined administration of adenovirus vector-based vaccines targeting RSV G and M2 proteins. a Schematic design of adenoviral vectors encoding RSV G and M2 antigens. (P_{CMV}, cytomegalovirus promoter; tPA, tissue plasminogen activator signal peptide; G (131–230), amino acid residues 131–230 of the G glycoprotein; mFcm, murine Fc receptor mutant; M2, open reading frame of the M2-1 protein). **b** Experimental timeline. Two experimental sets—a vaccination-only set and an RSV challenge set—were conducted in parallel. "D+number" indicates the number of days post-immunization. **c** Serum IgG titers against the G glycoprotein core fragment (GCF) were determined by ELISA 2 weeks after immunization. **d** GCF-specific IgA titers in BALF were measured by ELISA 3 weeks post-immunization. **e** Frequencies of M2-specific $CD8^+$ T cells in the lungs were analyzed using the $K^d/M2_{82-90}$ tetramer. **f** Frequencies of $CD69^+CD103^+$ tissue-resident memory T cells among M2-specific $CD8^+$ T cells were also assessed. **g**, **h** GCF-specific IgA titers in BALF were determined by ELISA 4 days after challenge with RSV A (**g**) or RSV B (**h**). **i**, **j** Frequencies of M2-specific $CD8^+$ T cells in the lungs were quantified following RSV A (**i**) or RSV B (**j**) infection using the $K^d/M2_{82-90}$ tetramer. **k**, **l** Frequencies of IFN-γ^+ $CD4^+$ T cells stimulated with the $G_{183-195}$ peptide (**k**) and IFN-γ^+ $CD8^+$ T cells stimulated with the $M2_{82-90}$ peptide (**l**) were analyzed in lung cells after RSV A infection. **m**, **n** The same analyses were conducted following RSV B infection. **o**, **p** RSV titers in the lungs were determined by plaque assay 4 days post-challenge and expressed as plaque-forming units (PFU) per gram of lung tissue. All data are presented as mean ± SD (n = 4–5 mice per group). Statistical significance was evaluated by one-way ANOVA with Tukey's multiple comparisons test. Significance levels: ns (not significant), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Dotted lines indicate the assay detection limits.

(Fig. 1f). Following RSV A or B challenge, GCF-specific IgA levels increased in both G and G + M2 groups with comparable titers (Fig. 1g–h). A robust increase in M2-specific $CD8^+$ T cells was observed post-infection in both M2 and G + M2 groups (Fig. 1i–j). Analysis of cytokine levels in the supernatant from homogenized lung tissue revealed a significant elevation of IFN-γ exclusively in the G + M2 group (Supplementary Fig. 3). Intracellular cytokine staining (ICS) showed that this IFN-γ production predominantly originated from M2-specific $CD8^+$ T cells upon peptide stimulation (Fig. 1k–n), consistent with the strong $CD8^+$ T cell–priming capacity of both the adenoviral vector and the M2 antigen[11,12]. Protective efficacy was evaluated by plaque assays. Following RSV A challenge, both G and G + M2 groups showed complete viral clearance, while the M2 group conferred minimal protection (Fig. 1o). In the RSV B challenge, although complete protection was not achieved—possibly due to both vaccine antigens being derived from the RSV A2 strain—all vaccinated groups exhibited significantly reduced viral titers (Fig. 1p). The inclusion of the M2 component was associated with enhanced viral reduction. These trends were consistently supported by RT-qPCR results (Supplementary Fig. 4a–b).

To further evaluate the protective efficacy of the vaccine while minimizing the impact of nonspecific innate immune responses elicited by the adenoviral vector, RSV challenge was conducted 3 months post-immunization (Fig. 2a). GCF-specific IgA levels in BALF remained high in the G + M2 group, indicating sustained mucosal immunity (Fig. 2b, c). Although the frequency of M2-specific $CD8^+$ T cells declined relative to earlier time points, substantial numbers persisted in the lungs (Fig. 2d, e). Viral load analysis using plaque assay showed complete protection against RSV A, with undetectable infectious virus in lungs from G + M2-vaccinated mice (Fig. 2f). During RSV B challenge, viral titers in the G + M2 group were significantly lower than those in mock controls (Fig. 2g), as supported by RT-qPCR data (Supplementary Fig. 4c, d). Body weight was monitored daily for 4 days following RSV challenge. The results showed minimal weight loss in the G + M2 group, compared to significant weight reductions observed in the mock group (Fig. 2h, i). To assess changes in innate immune cell populations in the BAL, we analyzed eosinophils, neutrophils, and alveolar macrophages by flow cytometry. Eosinophils were undetectable in all groups, and neutrophil levels showed no significant differences between the mock and G + M2 groups. Although the proportion of alveolar macrophages was significantly reduced in the G + M2 group, the absolute number of these cells remained similar. likely due to the increased accumulation of immune cells, especially lymphocytes, following vaccination (Supplementary Fig. 5). Histological analysis of lung sections showed moderate perivascular immune cell infiltration, primarily consisting of lymphocytes, in both the mock and vaccinated groups. No signs of bronchiolar inflammation or pneumonia were observed, and the degree of infiltration was comparable between the two groups (Supplementary Fig. 6). These results suggest that the G + M2 vaccine does not induce adverse immunopathology.

In summary, intranasal administration of the G and M2 vaccine combination elicited robust antibody and $CD8^+$ T-cell responses. This balanced immune profile provided effective protection against both RSV A and B. Nevertheless, the clinical translation of this dual-component vaccine presents several challenges. Determining the interactions between the two vaccines and optimizing their dosages is more complex than evaluating a single-antigen formulation. Furthermore, assessing tissue-resident T cells, primarily induced by the M2 component, is challenging due to the infeasibility of direct lung tissue sampling[13]. Despite these obstacles, our results suggest that the combination of RSV G and M2 vaccines is a promising candidate capable of inducing potent and broad immunity against both RSV strains.

Methods

Western blot

To confirm antigen expression and secretion, HEK293 cells were infected with adenoviral vector vaccines at a multiplicity of infection (MOI) of 10 in serum-free Minimum Essential Medium (MEM). After 2 days of incubation, cells were detached using a cell lifter and centrifuged at 1000 × g for 10 min at 4 °C to collect the culture supernatant. The supernatant was concentrated from 3 mL to ~100 µL using an Amicon® Ultra-4 Centrifugal Filter Unit (10 kDa MWCO; Merck Millipore, Carrigtwohill, Ireland). The concentrated samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% skim milk powder (blocking buffer), and incubated overnight at 4 °C with a mouse anti-GCF primary antibody diluted 1:2000 in blocking buffer. After washing, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG secondary antibody (Abcam, Cambridge, UK) diluted 1:5000 in blocking buffer. Protein bands were visualized using the Amersham™ ECL™ Prime Western Blotting Detection Reagent (Cytiva, Little Chalfont, UK) and detected using the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA).

Immunization and challenge

The Institutional Animal Care and Use Committee approved the animal experiments in this study (approval number 21-074-t), and all animals were housed under specific pathogen-free conditions at Ewha Womans University. BALB/c mice (Orient Bio Inc., Seongnam, Republic of Korea), aged 6 weeks, were placed in a chamber and anesthetized with isoflurane (Hana Pharm. Co. Ltd., Seoul, Republic of Korea) via a connected vaporizer (Midmark Corporation, Versailles, OH, USA) for a few minutes until the heart rate stabilized. Then 50 µL of vaccines were administered intranasally; 1×10^7 PFU rAd-M2 (M2), 3×10^7 PFU rAd-3xGmFcm (G), a mixture of 3×10^7 PFU rAd-3xGmFcm and 1×10^7 PFU rAd-M2 (G + M2, 3:1), or a mixture of 3×10^7 PFU rAd-3xGmFcm and 3×10^7 PFU rAd-M2 (3:3). The mice in the negative control group received 50 µL PBS or 4×10^7 PFU rAd-mock in 50 µL PBS (mock) intranasally. The mice were challenged with 1×10^6 PFU of RSV A2 or RSV B (human RSV-B isolate, KR/B/10-12)[14] diluted in 50 µL PBS following inhalation anesthesia with isoflurane using a

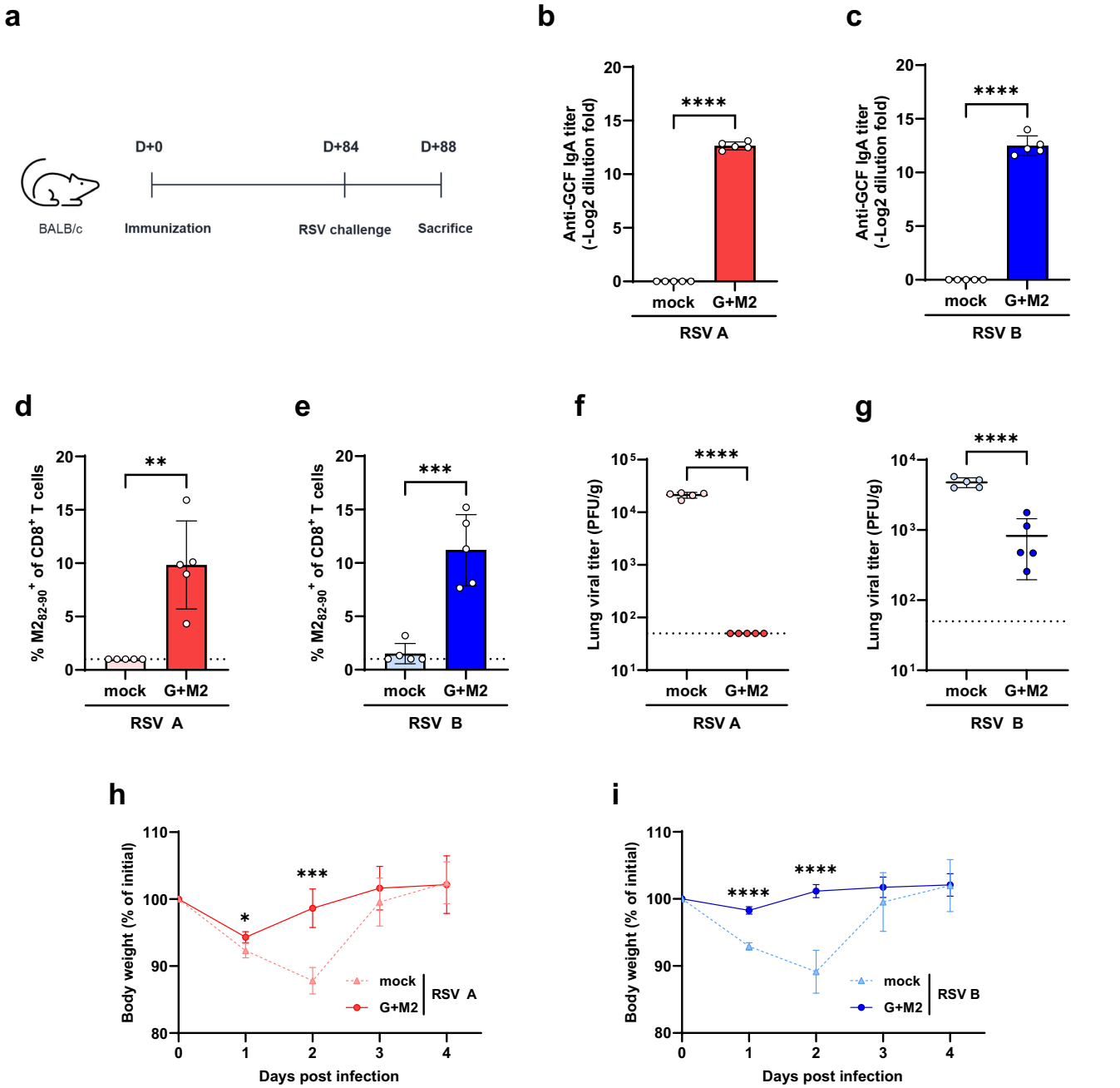


Fig. 2 | Evaluation of vaccine-induced protection 3 months post-immunization. **a** Experimental timeline. BALB/c mice were challenged with RSV A or RSV B at day 84 post-immunization and sacrificed four days later. **b**, **c** GCF-specific IgA titers in BALF were measured by ELISA following RSV A (**b**) or RSV B (**c**) challenge. **d**, **e** Frequencies of M2-specific CD8^{+} T cells in lung tissue were assessed following RSV A (**d**) or RSV B (**e**) infection using the K^{d}/M2$_{82-90}$ tetramer. **f**, **g** RSV titers in the lungs were determined by plaque assay following RSV A (**f**) or RSV B (**g**) infection and expressed as PFU per gram of lung tissue. **h**, **i** Body weight changes were monitored for 4 days following RSV A (**h**) or RSV B (**i**) challenge and are presented as percentages of initial body weight. All data are presented as mean ± SD ($n = 5$ mice per group). Statistical significance was assessed using an unpaired two-tailed Student's *t*-test. Significance levels: ns (not significant), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

vaporizer (L.M.S. KOREA, Seongnam, Republic of Korea). The mice were sacrificed 4 days later by over-anesthesia with isoflurane until breathing and heartbeat ceased. The preparation methods for adenovirus vector vaccine were described in detail in a previous study[15] and are summarized as follows. The rAd-3xGmFcm vector was generated using a synthetic DNA construct in which the G protein sequence from the RSV A2 strain was codon-optimized. The rAd-M2 vector was constructed by subcloning a PCR-amplified open reading frame (ORF) of the M2-1 protein from the RSV A2 genome, without codon optimization. RSV stocks were prepared as previously described[16].

Serum and Bronchoalveolar Lavage Fluid (BALF) collection

Blood was collected from the mice and allowed to stand at room temperature (RT) for at least 30 min to facilitate clotting. Subsequently, the blood was centrifuged at 5800 rpm for 15 min at 4 °C. The supernatant, which was the serum, was collected. BALF was obtained by centrifuging BAL at 4 °C and 5800 rpm for 15 min. The resulting supernatants were used.

Enzyme-linked Immunosorbent Assay (ELISA)

The GCF protein was diluted in PBS and 200 ng/well was added to 96-well immunoplates coated with MaxiSorp (Nunc, Roskilde, Denmark)

and incubated at 4 °C overnight. The preparation of the GCF protein has been previously detailed[17]. Afterwards, the protein-coated plates were washed three times with PBS containing 0.05% Tween-20 (PBST), followed by a 2-h blocking process with 200 µl of 1% skim milk in 0.05% PBST (blocking buffer) at RT. The serum and BALF samples were diluted with blocking buffer and incubated for 2 h at RT. HRP-conjugated rabbit anti-mouse IgG (Abcam, Cambridge, UK) or goat anti-mouse IgA (Abcam, Cambridge, UK), diluted to 1:2000 or 1:3000, respectively, was used as the secondary antibody and incubated for 1 h at RT. Subsequently, 3,3′,5,5′-tetramethylbenzidine substrate and solution (SeraCare, Milford, MA, USA) were added and incubated for 10–15 min at RT. The reaction was stopped using 1 M phosphoric acid, and absorbance was measured at 450 nm using a Thermo Multiskan EX plate reader (Thermo Fisher Scientific™, Waltham, MA, USA). The result of the ELISA is expressed as an endpoint titer, which indicates the dilution required to reach the absorbance mean of the blank plus three times its standard deviation.

Lung cell isolation

Before the lungs were harvested, they were perfused with PBS containing heparin at a concentration of 10 U/ml. The isolated lungs were placed in a 10% RPMI solution, which is a mixture of RPMI 1640 medium with 10% FBS, collagenase type IV at 20 U/ml and deoxyribonuclease I at 100 µg/ml. They were chopped with scissors and then incubated for 30 min at 37 °C, with the container inverted every 10 min. The mixture was then passed through a 70 µm cell strainer to separate single cells, followed by red blood cell lysis.

Tetramer staining and Tissue-Resident Memory T cell (TRM) analysis

Isolated lung cells were washed with flow cytometry staining buffer (PBS containing 0.5% fetal bovine serum and 0.1% sodium azide), then incubated with anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA, USA) to block Fc receptors, and with streptavidin (final concentration: 50 µg/mL) to prevent nonspecific binding of the tetramer. All staining and washing steps were performed using the same staining buffer. Subsequently, cells were stained with fluorochrome-conjugated anti-mouse CD3, CD8, CD69, CD103 antibodies (BioLegend, San Diego, CA, USA) and homemade PE-conjugated $K^d/M2_{82\text{-}90}$ tetramer. To identify TRM, 200 µL of PBS containing 2 µg of PerCP/Cyanine5.5 anti-mouse CD45 antibody (BioLegend, San Diego, CA, USA) was injected intravenously via the tail vein prior to euthanasia, allowing for the discrimination of circulating versus tissue-resident immune cells. Among the tetramer-positive population, $CD69^+CD103^+$ double-positive cells were defined as TRM. Following staining, cells were fixed using BD FACS™ Lysing Solution (BD Biosciences, Milpitas, CA, USA) and flow cytometry analysis was performed using a CytoFLEX S flow cytometer (Beckman Coulter, San Jose, CA, USA). Acquired data were analyzed with FlowJo software (Becton, Dickinson & Company, Ashland, OR, USA).

Intracellular Cytokine Staining (ICS)

Lung cells were incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 50 µM β-mercaptoethanol, Brefeldin A (1:1000 dilution; Thermo Fisher Scientific, San Diego, CA, USA), and either RSV $G_{183–195}$ or $M2_{82–90}$ peptide (final concentration: 10 µg/mL) at 37 °C for 5 h. Following stimulation, Fc receptors were blocked using anti-mouse CD16/CD32 antibody, and cells were stained for surface markers with fluorochrome-conjugated antibodies against CD3, CD4, and CD8. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, Milpitas, CA, USA), followed by intracellular staining with anti-IFN-γ antibody. Flow cytometry was performed using a CytoFLEX S flow cytometer (Beckman Coulter, San Jose, CA, USA), and data were analyzed with FlowJo software (Becton, Dickinson & Company, Ashland, OR, USA).

Plaque assay

The supernatant obtained after centrifugation of the lung cell suspension, which had been passed through a 70 µm cell strainer during cell isolation, was used to infect HEp-2 cells in 6-well plates for 2 h. The supernatant was then removed and overlaid with 4 mL of 0.5% agarose, which was mixed with 1% SeaPlaque® Agarose (Lonza, Rockland, ME, USA) dissolved in PBS and MEM containing 10% FBS. After incubation at 37 °C for 4–5 days and count the number of plaques was counted after further incubation with 2 ml/well overlaid with 0.5% agarose containing a neutral red solution (Sigma-Aldrich, Irvine, United Kingdom).

Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from the same samples used in the plaque assay using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. RT-qPCR was subsequently performed using the RealStar® RSV RT-PCR Kit 3.0 RUO (altona Diagnostics, Hamburg, Germany) according to the manufacturer's protocol. Amplification and fluorescence detection were carried out using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, USA). Quantification cycle (Cq) values were determined using the CFX Manager™ Software v3.1 (Bio-Rad, Hercules, CA, USA), based on the point at which the amplification curve crossed the threshold.

Quantification of cytokines

The cytokine concentrations present in the lung supernatants obtained in the same manner as the samples used for the plaque assay were quantified using a bead-based multiplex LEGENDplex™ assay (BioLegend, San Diego, CA, USA). The results were analyzed using LEGENDplex software provided by the company.

Bronchoalveolar Lavage (BAL) cells isolation

BAL was obtained by inserting a 20-gauge catheter (BD Angiocath Plus, Becton Dickinson Medical (s) Pte Ltd., Singapore) into the mouse bronchus and connecting a 1 ml syringe to inject and withdraw 1 ml of PBS three times before removing the lungs. The supernatant, BALF, was then removed by centrifugation at 4 °C and 5800 rpm for 15 min, and the resulting cell pellet was used.

Flow cytometric analysis of BAL cells

The isolated BAL cells were blocked with anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA, USA), stained with fluorochrome-conjugated antibodies against CD45, CD11c, SiglecF, and Ly6G (Gr-1) (BioLegend, San Diego, CA, USA), and then fixed with BD FACS™ Lysing Solution (BD Biosciences, Milpitas, CA, USA). Flow cytometry was performed using a CytoFLEX S flow cytometer (Beckman Coulter, San Jose, CA, USA), and data were analyzed with FlowJo software (Becton, Dickinson & Company, Ashland, OR, USA). BAL cell subsets were defined as follows: eosinophils ($CD45^+$ $CD11c^-$ $SiglecF^+$), alveolar macrophages ($CD45^+$ $CD11c^+$ $SiglecF^+$), and neutrophils ($CD45^+$ $CD11c^-$ $Ly6G^+$). The total number of BAL cells was determined using a Guava® Muse® Cell Analyzer (Luminex Corporation, Austin, TX, USA), and absolute cell counts for each subset were calculated by multiplying the percentage of gated populations by the total cell number.

Histopathology

Four days after RSV infection, the post-caval lobes of the lungs were excised and fixed in 10% neutral buffered formalin (HanLAB, Seoul, Republic of Korea), followed by sample preparation, hematoxylin and eosin (H&E) staining, and histopathological evaluation performed by KP&T Inc. (Cheongju, Republic of Korea). After fixation, the tissues were trimmed to a thickness of ~2–3 mm for histological processing. Trimmed specimens were placed into labeled cassettes and processed for 13 h using a tissue processor (STP120 Spin Tissue Processor, Myr). Subsequently, tissue sections were cut at a thickness of ~3–4 µm using a microtome (Finesse ME Microtome,

Thermo Shandon), mounted on glass slides, and air-dried. The slides were then subjected to deparaffinization, rehydration, and rinsing with distilled water. Finally, H&E staining was performed. Histopathological evaluation was conducted with reference to previously reported studies[18,19].

Statistical analysis

Data analysis and visualization were performed using Prism software (GraphPad Software, Boston, MA, USA). The results are shown as mean ± standard deviation (SD), and statistical significance was determined using an unpaired two-tailed Student's *t*-test or ordinary one-way ANOVA with Tukey's multiple comparisons test for two groups or more than two groups, respectively. Significance levels are denoted as follows: not significant (ns), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$

Data availability

The DNA sequence of the antigen insert used in the rAd-3xGmFcm has been deposited in GenBank under accession number PV623153. The insert sequence used in the rAd-M2 is derived from the RSV A2, available in GenBank under accession number KT992094.

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Author contributions

E.J. and J.C. conceived and designed the experiments. E.J., S.L., and J.H. performed the experiments. E.J. and J.C. analyzed the data. E.J. and J.C. wrote the paper. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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