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Article

Hypertonic intranasal vaccines gain nasal epithelia access to exert strong immunogenicity

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ABSTRACT

Intranasal vaccines potentially offer superior protection against viral infections compared with injectable vaccines. The immunogenicity of intranasal vaccines including adenovirus vector (AdV), has room for improvement, while few options are available for safe execution. In this study, we demonstrate that modifying a basic parameter of vaccine formulation, i.e., osmolarity, can significantly enhance the immunogenicity of intranasal vaccines. Addition of glycerol to AdV intranasal vaccine solutions, unlike other viscous additives, enhanced systemic and mucosal antibodies as well as resident memory T cells in the nasal tissues, which could protect nasal tissue and the lungs against influenza virus. While viscous glycerol could not prolong intranasal retention of solutes, it promoted AdV infection of nasal epithelial cells by facilitating AdV access to the nasal epithelial cell. The enhanced immunogenicity was induced by the hypertonicity of vaccine preparations and sodium chloride, glucose, and mannitol demonstrated the capacity to enhance immunogenicity. Moreover, hypertonic glycerol enhanced the immunogenicity of adjuvanted subunit intranasal vaccines, but not subunit vaccines without adjuvant or injectable vaccines. Overall, the delivery of intranasal vaccines to nasal epithelial cells could be improved through a simple approach, potentially resulting in stronger immunogenicity for certain vaccines.

Introduction

As the mucosal surface of the upper respiratory tract (URT) is the initial site of respiratory viral infection,[1] antibodies and T cells induced in the URT can effectively prevent transmission.[2–4] Thus, intranasal vaccines that immunize the URT to induce robust mucosal immune responses have emerged as promising candidates for improved vaccines.[5]

The mucosal surface is coated with mucus gel, which is propelled atop the periciliary layer (PCL); this consists of a macromolecular mesh tethered to either cilia, microvilli, or epithelial surface.[6] Intranasally administered vaccines are prone to rapid entrapment in the mucus and subsequently washed out by cilia-generated forces.[7] Meanwhile, the PCL prevents the vaccines and mucus from penetrating the epithelial cell layer.[6] Thus, antigen absorption to the body or even antigen access to the cell surface are low followed by intranasal administration, resulting in a challenge of low immunogenicity of the intranasal vaccines.[8] Furthermore, with the potential side effects of an adjuvant used in the inactivated influenza vaccine (i.e., Bell's palsy), an effective adjuvant with a proven safety profile is still not available.[8,9] In this context, prolonging intranasal vaccine retention against this mucociliary

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clearance, by using safe mucoadhesive, such as viscous additives or gel-forming polymers, has shown promise in improving immunogenicity of subunit vaccines.[10–12] Another approach is viral vectors coding vaccine antigens that utilize their natural tropism to respiratory epithelium for intracellular delivery of coding antigens.[9] Although the mucosal barrier remains effective against viral vector infection,[13,14] their intrinsic adjuvanticity, coupled with the lack of proven mucosal adjuvants, positions them among the most popular candidates for intranasal vaccines in clinical trials.[8]

Adenovirus vectors (AdV) are expected for intranasal vaccines owing to their natural tropism to URT, proven tolerability in parental vaccines against SARS-CoV-2, genetic malleability, and intrinsic immunogenicity,[15,16] confirmed as promising immunogenicity in pre-clinical models.[17–20] However, recent reports from clinical trials have indicated low immunogenicity of intranasal AdV, with instances of undetectable or minimal induction of antibodies in the URT.[21–23] Potential reasons for this discrepancy in the preclinical model remain elusive. In addition, the poor immunogenicity of AdV might raise the challenge of safe improvement of the immunogenicity of intranasal vaccines; further, whether mucoadhesive approaches work with AdV remains to be tested.

In this study, we observed that a low-volume intranasal AdV vaccine, immunizing only the URT and not the lungs, had low immunogenicity, even in mice. Using this intranasal AdV vaccine model, we screened several commonly used viscous additives to safely enhance the immunogenicity of intranasal AdV vaccines. Our findings revealed that glycerol, but not others, significantly improved the immunogenicity of intranasal AdV. Notably, glycerol was not found to be mucoadhesive; rather, its role in mediating vaccine hypertonicity enhanced intranasal AdV immunogenicity. Consistently, preparations of hypertonic AdV vaccines using sodium chloride, glucose, or mannitol also recapitulated the increased immunogenicity. Additionally, hypertonic glycerol boosted the immunogenicity of an adjuvanted subunit intranasal vaccine. Thus, we propose a non-adhesive approach to enhance the immunogenicity of intranasal vaccines with common, safe additives.

Results

Small-volume intranasal vaccine of AdV demonstrates weak immunogenicity in mice

Many murine intranasal vaccine models require administration of large volumes of vaccine-containing solutions (i.e., 20–30 μL/mouse) to immunize both the URT and lower respiratory tract (LRT)—including the lungs.[2,24] It is possible, however, that such approaches have resulted in discrepancies in the observed vaccine effects between murine studies[17–19] and clinical studies[21–23] using AdV intranasal vaccines. To evaluate the effect of different volumes of intranasal AdV vaccines, we intranasally treated mice with 5×10^8 inclusion forming units (IFUs) AdV carrying a model antigen ovalbumin (OVA) gene (AdV-OVA) in either 30 μL, to immunize the total respiratory tract (TRT; URT + LRT), or 6 μL, to limit vaccine delivery to URT.[2,24] Twenty eight days post-immunization, we measured OVA-specific antibodies and $CD8^+$ T cell responses in the blood, nasal passage, and lungs to assess systemic and mucosal immune responses (Fig. 1 and Supplementary Fig. 1). We detected OVA-specific IgG in the plasma of the URT group compared with the naive mice control, although the levels were approximately 100 times lower than those in the TRT group, or intramuscularly immunized (IM) group, which was used as a positive control (Fig. 1A). Significant levels of OVA-specific IgA were observed in nasal washes solely in the TRT group (Fig. 1B). OVA-specific IgG was detected in the bronchoalveolar lavage fluid (BALF) in both the TRT and IM groups, but not in the URT group, while OVA-specific IgA was detected in the BALF in only the TRT group (Fig. 1C). Thus, only large volume of intranasal AdV vaccine (i.e., TRT) induced comparable systemic antibody responses in the IM group and significantly higher mucosal antibody responses in both the URT (the nasal passage) and LRT (the lungs).

OVA-specific $CD8^+$ T cells were detected using flow cytometry with H-2 Kb $OVA_{257\text{-}264}$ tetramer (OVAtet). We also performed intravenous staining of CD45 to distinguish $OVAtet^+CD8^+$ T cells in the vasculature and tissue parenchyma and evaluated the CD45 i.v.$^-$$OVAtet^+CD8^+$ T cells for CD69 and CD103 to identify tissue-resident memory T cells (Trm)[24,25] in the URT (Fig. 1D–K) and lungs (Supplementary Fig. 1). OVA-specific $CD8^+$ T cells were not found in the blood but were significantly detected in the nasal tissue following URT immunization, compared with the naive group (Fig. 1D and E). Additionally, $CD8^+$ T cells in the nasal tissue were observed in the TRT and IM groups, but a lower frequency of CD45 i.v.-negative staining was observed in the IM group; further, CD103 expression on the CD45 i.v.$^-$ OVA-specific $CD8^+$ T cells were almost exclusively noted in the URT and TRT groups (Fig. 1F–K). These results suggest that URT and TRT treatments successfully immunized the nasal tissue and induced Trm cells, which is consistent with the requirement of local immunization for Trm cell induction in nasal tissue.[24] In contrast, although OVA-specific $CD8^+$ T cells were significantly detected in the lungs of both the TRT and IM groups (Fig. 1D and E), only those in the TRT group induced significant number of $CD69^+CD103^+$CD45 i.v.$^-$$OVAtet^+$ Trm cells (Supplementary Fig. 1). Thus, TRT efficiently induced Trm in the lungs, whereas URT and IM did not. Given that lung Trm induction requires lung local antigen,[26] TRT, but not URT, could deliver AdV or AdV-derived antigen to the lungs, consistent with our previous observations of intranasal subunit vaccines.[2] Thus, intranasal vaccination with a small volume of AdV targeting the URT was not immunogenic in mice.

Effect of glycerol in intranasal small volume AdV vaccine

Considering that increased intranasal retention of subunit vaccines can enhance immunogenicity by promoting antigen absorption through the mucosal barrier,[10,11,27] we hypothesized that additives that are often used as thickeners could boost AdV retention in the nasal passage, thereby promote infection and result in improved immunogenicity. We prepared a mixture of each additive and AdV in phosphate-buffer saline (PBS) and intranasally immunized mice with 6 μL volume. The doses of additives tested were determined based on their actual concentrations used in medicines and our pre-examinations of their dose–response, with the top concentration of each additive being approximately the maximum limit of water solubility (Fig. 2A and B). We found that adding 10 % glycerol increased intranasal AdV immunogenicity by nearly 100 times in terms of plasma OVA-specific IgG 28 days after immunization (Fig. 2C). Mucosal antibody responses mediated by OVA-specific IgA in nasal wash were also significantly enhanced by addition of 10 % glycerol, compared with the PBS control (Fig. 2D). However, the effect of glycerol in increasing AdV immunogenicity reached a plateau at 10 % concentration (Fig. 2A and B). Although OVA-specific $CD8^+$ T cells in blood did not increase, those in nasal tissues were significantly amplified by 10 % glycerol (Fig. 2E and F). The other additives tested did not enhance the immunogenicity of intranasal AdV. Thus, we conclude that addition of glycerol to an intranasal AdV vaccine could enhance its immunogenicity.

Glycerol increases AdV infection in nasal epithelial cells

We evaluated whether glycerol-induced increase in immunogenicity of intranasal AdV was due to heightened AdV infection in the nasal tissue. DNA was extracted from the nasal tissues from mice treated with AdV intranasally with either PBS, 10 % glycerol, or 0.55 % CVP. AdV genome copies in the DNA were measured using qPCR. Significant AdV genome copies were detected in the nasal DNA from mice treated AdV in PBS than in that from naive control. Furthermore, the addition of 10 % glycerol increased this amount by approximately 40-fold (Fig. 3A). Addition of 0.55 % CVP to the AdV did not increase AdV genome copies. This suggests that 10 % glycerol significantly promotes AdV infection in nasal tissue. Subsequently, we evaluated if the increased infection led to antigen expression. Nasal sections were prepared from mice that were

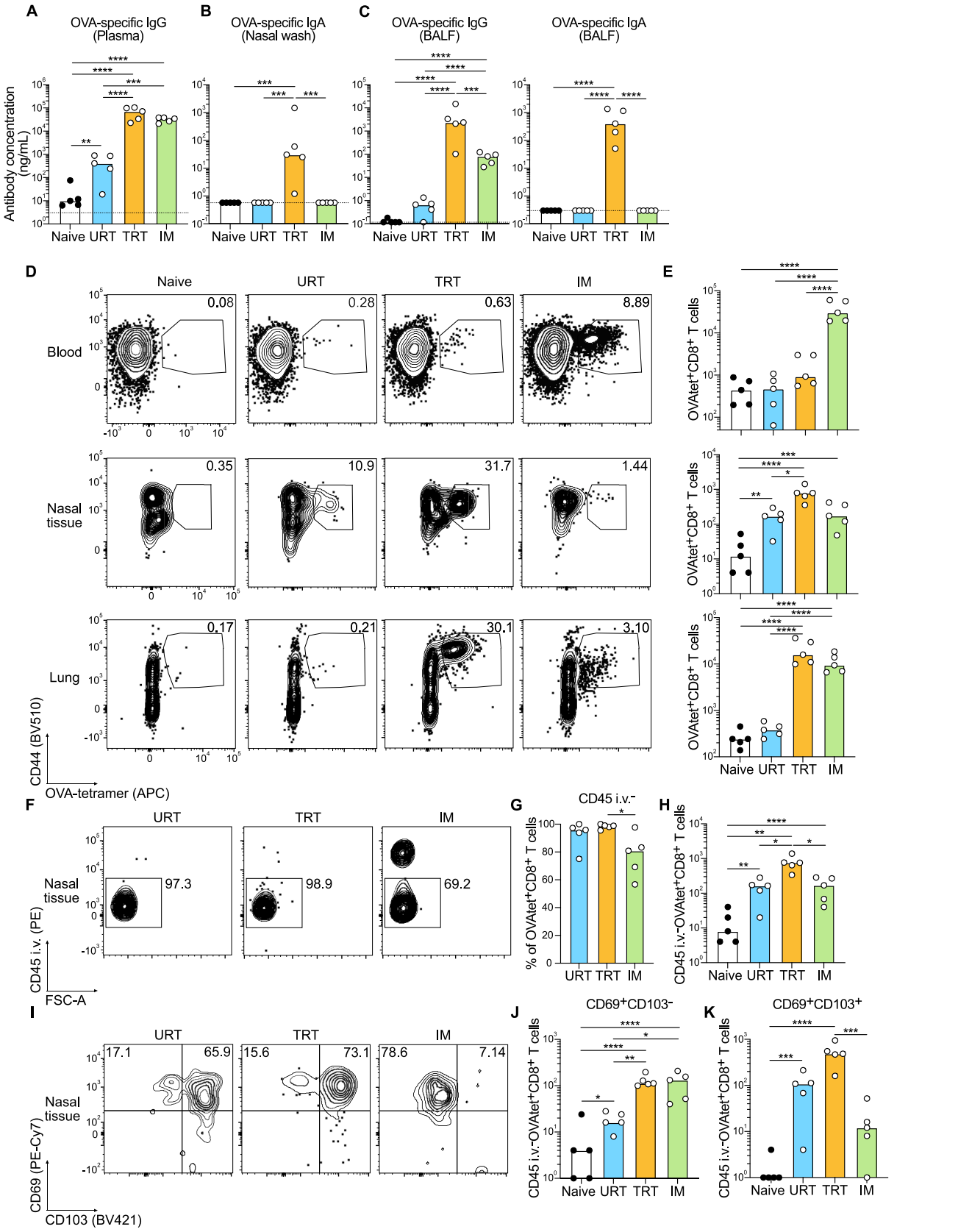

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Fig. 1. Small-volume intranasal AdV vaccine exerts weak immunogenicity in mice. C57BL/6J mice were intranasally administered with AdV-OVA (5×10^8 IFU/mouse) in a total volume of 6 μL (URT; upper respiratory tract, 3 μL/nostril) or 30 μL (TRT; total respiratory tract, 15 μL/nostril), or intramuscularly (IM; 20 μL/mouse). The following steps were conducted after 28 days of vaccination: intravenous injection of anti-CD45 antibody 5 min prior to blood sampling, nasal wash, bronchoalveolar lavage fluid (BALF), and nasal and lung tissue harvesting for subsequent assays. (A–C) OVA-specific IgG in plasma (A), OVA-specific IgA in nasal wash (B), and OVA-specific IgG (C, left) or IgA (C, right) in the BALF were evaluated using ELISA. Dotted lines indicate the limit of detection. (D and E) Representative flow cytometry plot gated on live $CD90^+CD8^+$ T cells (blood) or live $CD45^+CD90^+CD8^+$ T cells (nasal tissue and lung) (D) and the corresponding summary data (E) in the blood, nasal tissue, and lung. (F–H) Representative flow plot of CD45 intravascular staining (CD45 i.v.) gated on live $CD90^+OVAtet^+CD8^+$ T cells in nasal tissue (F) and the corresponding summary data of ratio (G) and number (H) of CD45 i.v.$^-$$OVAtet^+CD8^+$ T cells. (I–K) Representative flow plot gated on live CD45 i.v.$^-$$CD90^+CD8^+OVAtet^+$ T cells in nasal tissue analyzing for CD69 and CD103 expression (I) and the corresponding summary data of $CD69^+CD103^-$ (J) and $CD69^+CD103^+$ (K) CD45 i.v.$^-$$OVAtet^+CD8^+$ T cells. Data are representative of two separate experiments. Each symbol denotes data from an individual animal, and each bar represents the median except the panel in G. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ as determined by one-way ANOVA and Tukey's multiple comparisons test. See also Supplementary Fig. 1.

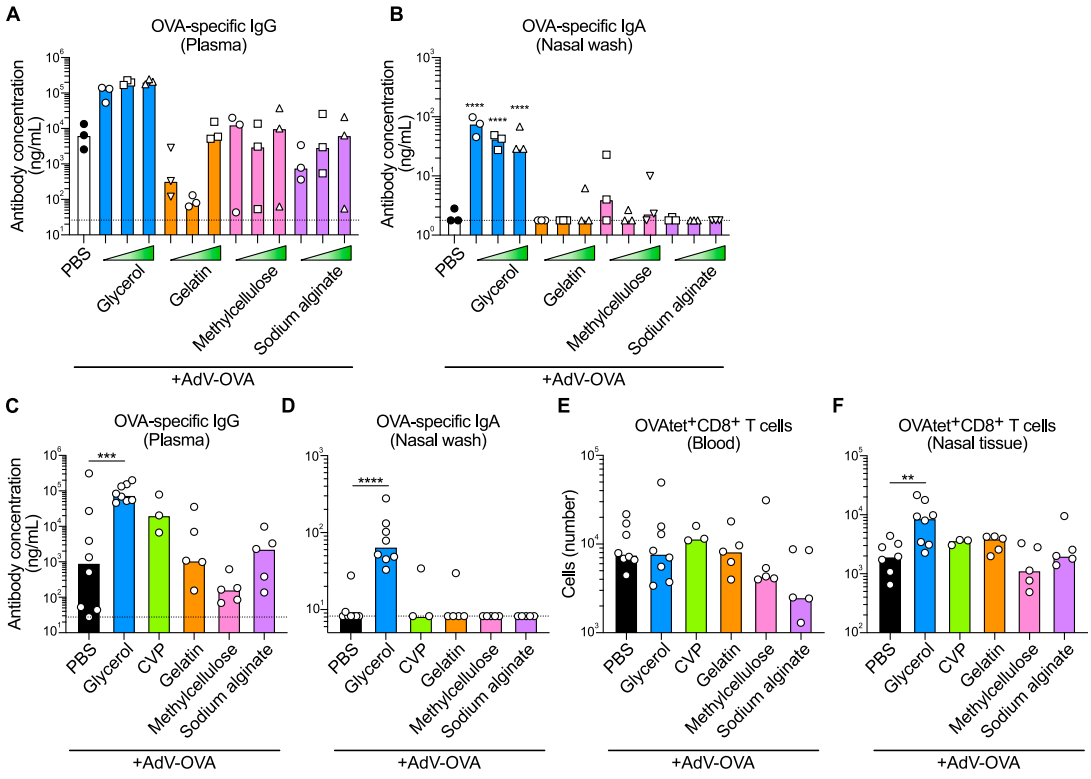

Fig. 2. Glycerol increases immunogenicity of intranasal AdV vaccines. (A–B) C57BL/6J mice intranasally administered with 6 μL of AdV-OVA (5×10^8 IFU/mouse) dispersed in 10 %, 30 %, or 90 % glycerol, 0.1 %, 1 %, or 10 % Gelatin, 0.002 %, 0.02 %, or 0.2 % methylcellulose or 0.01 %, 0.1 %, or 1 % sodium alginate in PBS. Twenty-eight days post-vaccination, blood and nasal wash samples were collected. OVA-specific IgG in plasma (A) and OVA-specific IgA in nasal wash (B) were evaluated using ELISA. Dotted lines indicate the limit of detection. (C–F) C57BL/6J mice were intranasally administered with 6 μL of AdV-OVA (5×10^8 IFU/mouse) dispersed in 10 % glycerol, 0.55 % CVP, 1 % gelatin, 0.02 % methylcellulose or 1 % sodium alginate in PBS. Twenty-eight days post-vaccination, blood, nasal wash, and nasal tissues were harvested. OVA-specific IgG in plasma (C) and OVA-specific IgA in nasal wash (D) were evaluated using ELISA. Dotted lines indicate the limit of detection. Live $CD90^+CD8^+OVAtet^+$ T cells in blood (E) and live $CD45^+CD90^+CD8^+OVAtet^+$ T cells (F) were enumerated in nasal tissue via flow cytometry. Data show pooled results of two separate experiments. Each symbol represents data from an individual animal and each bar denotes the median. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ as determined by one-way ANOVA and Dunnett's multiple comparisons test vs PBS group.

intranasally treated with AdV carrying enhanced GFP (AdV-GFP) in 6 μL PBS containing 10 % glycerol and stained for GFP (green), acetyl-a-tubulin (red), which is a marker of cilia on epithelial cell surface, and nucleus (blue) (Fig. 3B). GFP staining was minimal in the nasal tissues of mice treated with AdV-GFP in PBS, and was markedly increased by AdV-GFP in glycerol (Fig. 3B, upper panels). This staining was observed in the nasal turbinate, but not around nasal-associated lymphoid tissues (NALTs). The magnified images showed GFP-expressing cells lined just beneath acetyl-a-tubulin staining, suggesting that they were ciliated epithelial cells and that AdV rarely infects cells that are not exposed to nasal tissue surface. To further characterize the GFP-expressing cells, we analyzed the nasal tissue intranasally treated with AdV-GFP by flow cytometry. Most GFP^+ cells were detected in $CD45^-$ non-immune cells, and most of these $CD45^-GFP^+$ cells were Ep-CAM-positive epithelial cells (Fig. 3C and D). Therefore, glycerol increased intranasally administered AdV infection in nasal epithelial cells, which lead to augmented antigen expression.

To test the ability of glycerol to prolong intranasal solute retention, potentially owing to its viscous nature, we intranasally administered mice with recombinant luciferase in 10 % glycerol in PBS and measured luciferase activity in the nasal washes over time to evaluate intranasal retention of the luciferase (Fig. 3E). Addition of a viscous carboxy vinyl polymer (CVP) significantly increased luciferase activity in the nasal wash 4 h after administration compared with the control PBS, indicating that CVP increased intranasal retention of luciferase, which is consistent with previous reports.[10,28] In contrast, luciferase recovery in nasal wash was decreased 4 h post-administration in 10 % glycerol and did not increase at other time points as well. Thus, glycerol promoted nasal epithelial AdV infection, but possibly not because it increased the intranasal retention of AdV.

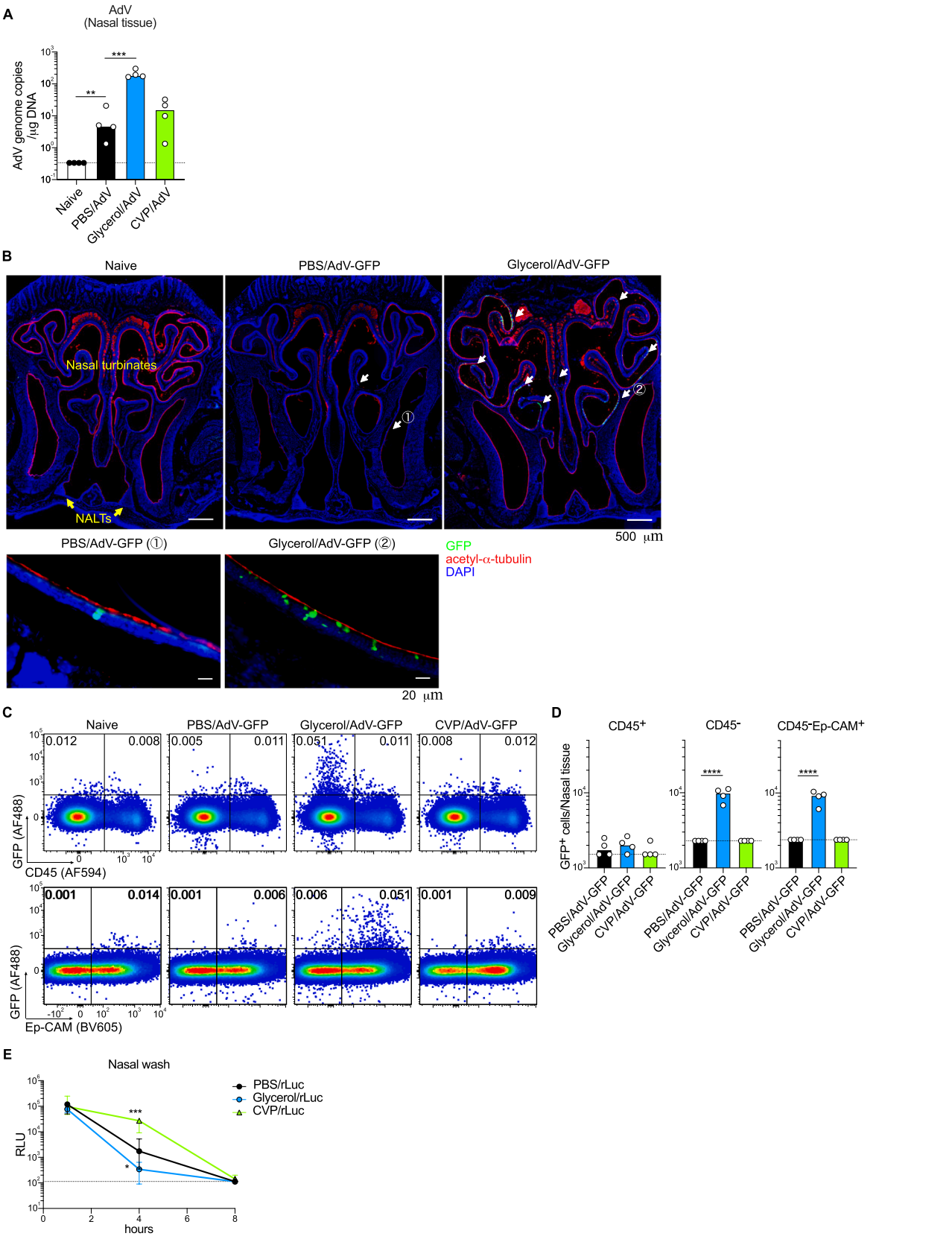

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Fig. 3. Glycerol increases AdV infection in nasal turbinate epithelial cells. (A) C57BL/6 mice were intranasally administered with AdV-OVA (5×10^8 IFU/mouse) in either 6 μL PBS or 10 % glycerol in PBS. Nasal tissue was harvested 24 h after the administration and AdV genomic copies in the nasal DNA were determined by qPCR. (B–D) C57BL/6 mice were intranasally administered with AdV-GFP (5×10^8 IFU/mouse) in either 6 μL PBS or 10 % glycerol in PBS. (B) Representative immunofluorescence images of nasal tissue harvested 3 days post-AdV treatment and stained for GFP (green) and acetyl-a-tubulin (red) and DAPI (blue). Lower panels display magnified images of the areas indicated in the upper panels. (C and D) Representative flow cytometry plot gated on live cells (upper panels), or live $CD45^-$ cells (lower panels) in nasal tissue (C) and their summary data (D) are shown. Dotted lines indicate the average of two naive C57BL/6 mice. (E) Recombinant luciferase (10 ng/mouse) were administered in a total of 6 μL PBS, 10 % glycerol, or 0.55 % CVP in PBS. Nasal washes were collected at 1, 4, and 8 h post-treatment, and luciferase activity was measured as relative luminescence unit (RLU). Dotted lines indicate the value of blank wells in the assay. Data are representative of two or more independent experiments (A–D) or pooled results of two separate experiments of n = 3 or 4 mice (E). Each symbol denotes data from an individual animal and each bar represents the median (A and D) or represents group median (E). Flow cytometry plots are shown as concatenations for all mice in each group within the same experiment. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as determined by one-way ANOVA (A and D) or two-way ANOVA (E) and Dunnett's multiple comparisons test vs PBS group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Hypertonic preparations with glycerol promote nasal epithelial access of intranasal vaccines and enhance immunogenicity

Glycerol is a small molecule, and its addition has a significant effect on the osmolality of vaccines and thus on mucus layer upon intranasal administration. The high osmolarity of the mucus layer results in the temporal collapse of the PCL, which has been shown to be the major barrier against AdV infection in airway epithelial cells.[6,13] Therefore, we hypothesized that osmolarity is the mechanism behind the enhanced intranasal AdV immunogenicity by glycerol. We used 10 % glycerol in PBS, whose total osmolarity was approximately five times more than isotonic, given that 2.5 % glycerol in water is isotonic and PBS itself is isotonic (Fig. 4A). We found that intranasal AdV vaccines in 2.5 % glycerol in water showed low immunogenicity, similar to that of intranasal AdV vaccines in water, while those in 5 % or 10 % glycerol in water or PBS significantly increased the immunogenicity (Fig. 4B–D). This suggests that preparations of 6 μL intranasal AdV vaccine with twice the osmotic pressure of isotonic solutions are highly immunogenic. We then tested whether hypertonic intranasal AdV vaccines prepared using additives other than glycerol also exhibited high immunogenicity. All AdV preparations of 2.5 % glycerol, 0.9 % sodium chloride, 5 % glucose, or 5 % mannitol in PBS, which were twice the osmotic pressure of isotonic (Fig. 4A), showed greatly enhanced AdV immunogenicity (Fig. 4E–G). Furthermore, more than twice the osmotic pressure of isotonic did not further increase immunogenicity in our model. Notably, hypotonic AdV in water did not increase immunogenicity compared with isotonic AdV in PBS. Based on these data, we conclude that hypertonic preparations of intranasal AdV vaccines can result in strong immunogenicity.

To determine whether hypertonic glycerol improves access of solutes, including AdV, to the epithelial surface, anti-Ep-CAM antibodies in 10 % glycerol were intranasally administered to mice, and nasal tissue was collected to examine epithelial cells to which the anti-Ep-CAM bound. Higher amounts of anti-Ep-CAM bound to $CD45^-CD31^-$ epithelial cells were detected following intranasal treatment with anti-Ep-CAM in 10 % glycerol than in PBS (Fig. 4H and I). To test whether 10 % glycerol increases the permeability of the epithelial barrier rather than increasing epithelial access, mice were intranasally treated with FITC-dextran in PBS, 10 % glycerol in PBS, or 1 % capric acid, which is an absorption enhancer, as the positive control.[29] The results showed that 10 % glycerol did not enhance the absorption of FITC-dextran detected in plasma, whereas 1 % capric acid significantly increased absorption compared, with the vehicle control, indicating 10 % glycerol did not promote absorption (Fig. 4J). Therefore, we summarize that intranasally administered hypertonic glycerol provides solutes with better access to nasal epithelial cell surface, which allows AdV to infect epithelial cells more effectively.

Hypertonic solution shows low inflammatory property in nasal tissue with optimal osmotic pressure

To evaluate the inflammatory nature of hypertonic solutions that could result in adjuvanticity, nasal tissue sections were analyzed through hematoxylin and eosin staining 24 h after intranasal administration of 10 % glycerol in PBS with or without AdV (Fig. 5A and Supplementary Fig. 2). We did not observe signs of tissue inflammation, such as inflammatory cell infiltrate in mice treated with glycerol, even with AdV, which was also confirmed by flow cytometry (Fig. 5B). mRNA expression of a set of inflammatory cytokines in nasal tissues was further evaluated by qPCR 4 and 24 h after 10 % glycerol in PBS intranasal administration with/without AdV (Fig. 5C). Notably, although 10 % glycerol did not increase most of mRNA levels of inflammatory cytokines that we evaluated (i.e. *Il1a*, *Il1b*, *Il12p40*, *Il33*, *TNFa*, *Infa*, *Infb*, and *Ifng*), we observed that *Il6* levels increased 4 h after intranasal administration of 10 % glycerol, but returned to a steady state in 24 h (Fig. 5C). *Il6* levels were also increased by intranasal administration of 3.6 % NaCl in PBS (Fig. 5D), suggesting that hypertonicity rather than the nature of glycerol could be the mechanism of its temporal increase. We also observed hypertonicity-dependent induction of *Il6* expression (Fig. 5D). However, immunogenicity-enhancing 2.5 % glycerol in PBS did not significantly induce *Il6* (Fig. 4E–G). Notably, *Il1b*, *Il12p40*, *Infb*, and *Ifng* significantly increased 24 h after intranasal administration in the glycerol/AdV group, but not in the glycerol-only group (Fig. 5C). Increased levels of these cytokines are known to be induced during AdV-infection,[30] suggesting that glycerol appeared to increase AdV infection, which, rather than hypertonicity, led to increased mRNA expressions. Taken together, intranasal hypertonic solution are not highly inflammatory depending on their hypertonicity, yet they promote AdV infection, thereby enhancing immunogenicity.

Hypertonic intranasal AdV vaccine enhances protective immunity

To evaluate whether the glycerol-mediated enhanced AdV immunogenicity was also observed with other antigens (i.e., spike protein from SARS-CoV-2, Wuhan-Hu-1 strain: S, or nucleoprotein from type A influenza viruses: NP, AdV-S, and AdV-NP respectively), we immunized mice intranasally with AdV-S or AdV-NP 6 μL in PBS containing 10 % glycerol (Supplementary Fig. 3 and Fig. 6). Intranasal AdV-S in PBS (vehicle) significantly induced S-specific IgG in the plasma, compared with the naive control, which was significantly enhanced by adding glycerol (Supplementary Fig. 3A). Mucosal IgA responses, which were poor in reported clinical trials, were not detected in mice treated with AdV-S in the vehicle but were significantly detected in mice treated with glycerol (Supplementary Fig. 3B). The number of spike-specific $CD8^+$ T cells detected by H-2 Kb $S_{539\text{-}546}$ tetramer (Stet) were increased in the nasal passage, but not significantly in the blood in AdV-S-treated groups either in PBS or glycerol, compared with the naive control (Supplementary Fig. 3C and D). We also evaluated the neutralizing capacity of S-specific antibodies in the serum and nasal washes using vesicular stomatitis virus-based pseudotyped viruses expressing spike proteins from the Wuhan-Hu-1 strain with a glycine substitution at position 614 (D614G) or Delta variant of SARS-CoV-2. We observed that serum from the Glycerol/AdV-S group neutralized pseudotyped viruses expressing either D614G or delta spike of SARS-CoV-2 significantly better than did the serum from PBS/AdV-S group (Supplementary Fig. 3E and F). A slight trend of neutralization by nasal washing from

A

Solute	Vehicle	Osmotic pressue
non	PBS	isotonic
2.5% Glycerol	water	isotonic
0.9% NaCl	water	isotonic
5% Glucose	water	isotonic
5% Mannitol	water	isotonic
5% Glycerol	water	hypertonic (2×isotonic)
2.5% Glycerol	PBS	hypertonic (2×isotonic)
0.9% NaCl	PBS	hypertonic (2×isotonic)
5% Glucose	PBS	hypertonic (2×isotonic)
5% Mannitol	PBS	hypertonic (2×isotonic)
10% Glycerol	water	hypertonic (4×isotonic)
10% Glycerol	PBS	hypertonic (5×isotonic)
3.6% NaCl	PBS	hypertonic (5×isotonic)
20% Glucose	PBS	hypertonic (5×isotonic)
20% Mannitol	PBS	hypertonic (5×isotonic)

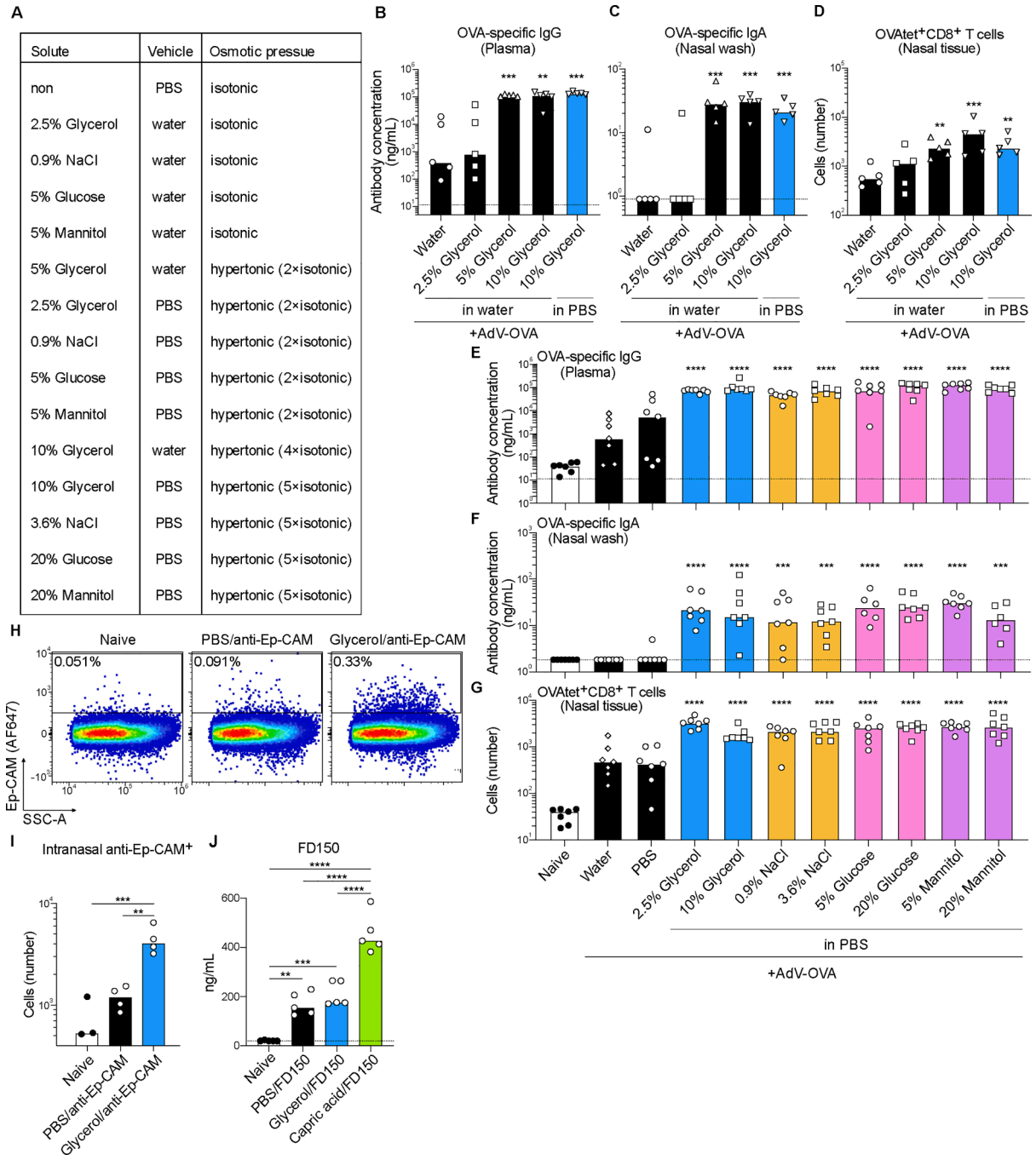

Fig. 4. Hypertonic preparation promotes nasal epithelial access of solutes and enhances AdV immunogenicity. (A) A quick guide of the vaccine preparations and their rough osmotic pressure compared to isotonic vaccines. (B–D) C57BL/6 mice were intranasally immunized with AdV-OVA (1×10^8 IFU/mouse) in a total of 6 μL water, glycerol-added water, or PBS at different concentrations as indicated. Twenty-eight days post-immunization, plasma, nasal wash, and nasal tissue were harvested. OVA-specific IgG in plasma (B) and OVA-specific IgA in nasal wash (C) were evaluated using ELISA. Dotted lines indicate the limit of detection. Live $CD45^+CD90^+CD8^+OVAtet^+$ T cells (D) in nasal tissue were enumerated via flow cytometry. (E–G) C57BL/6 mice were intranasally immunized with AdV-OVA (1×10^8 IFU/mouse) in a total of 6 μL water, PBS, glycerol, sodium chloride (NaCl), glucose, or mannitol-added PBS. After 28 days of immunization, plasma, nasal wash, and nasal tissue were harvested. OVA-specific IgG in plasma (E) and OVA-specific IgA in nasal wash (F) were evaluated using ELISA. Dotted lines indicate the limit of detection. Live $CD45^+CD90^+CD8^+OVAtet^+$ T cells (G) in nasal tissue were enumerated via flow cytometry. (H and I) C57BL/6 mice were intranasally administrated with 0.1 μg of anti-Ep-CAM antibody in 6 μL PBS, 10 % glycerol, or 0.55 % CVP in PBS. Nasal tissues were harvested 2 h post-administration to detect the intranasally treated anti-Ep-CAM antibodies binding to epithelial cells. Representative plots of nasal tissues gated on live $CD45^-CD31^-$ epithelial cells (H) and their summary data (I). (J) C57BL/6 mice were intranasally administrated 1.25 mg FD150 in PBS or 10 % glycerol in PBS. Plasma concentrations of FD150 were determined 1 h after the treatment. Dotted lines indicate the limit of detection. Each symbol represents data from an individual animal. Bars indicate median. Data are representative (B–D and H–J) or pooled data (E–G) of two separate experiments. Flow cytometry plots are shown as concatenations for all mice in each group within the same experiment. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as determined by one-way ANOVA and Dunnett's multiple comparisons test vs water or PBS (B to G), or Tukey's multiple comparisons test (I and J).

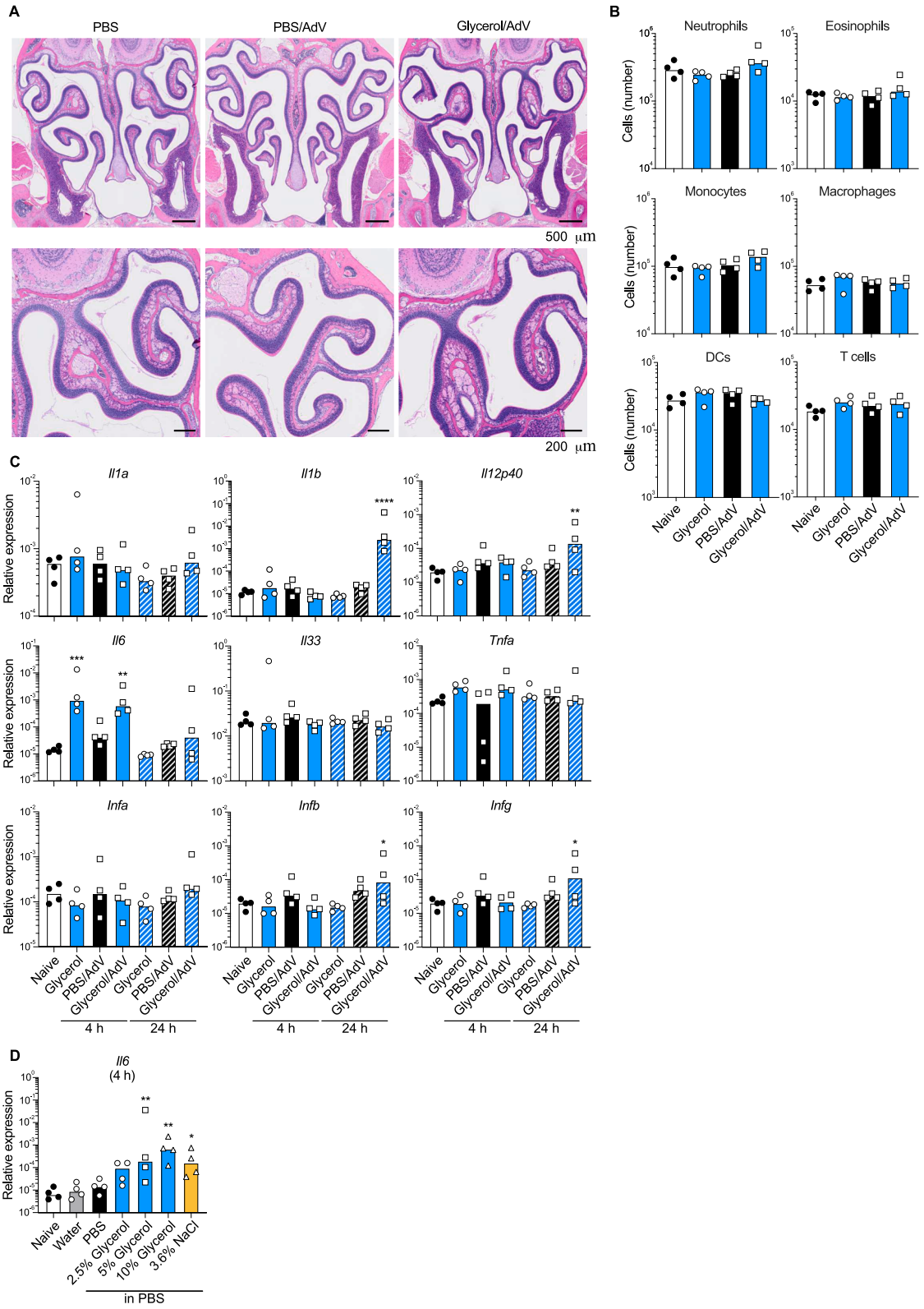

Fig. 5. Inflammatory properties of hypertonic intranasal AdV vaccines. (A–C) C57BL/6J mice intranasally treated with 6 μL of PBS, 10 % glycerol in PBS, AdV-OVA (5×10^8 IFU/mouse) in PBS, or AdV-OVA (5×10^8 IFU/mouse) in 10 % glycerol in PBS. (A) Representative hematoxylin and eosin stained histological images of nasal tissues 24 h after the treatments. Upper and lower panels display the same sample at different magnifications. (B) Inflammatory cell infiltrates in nasal tissues were enumerated by flow cytometry 24 h after the treatments. (C) mRNA expression levels of inflammatory cytokine normalized to *Gapdh* in nasal tissue at the specified time after the intranasal treatment. (D) Water, PBS, various doses of glycerol in PBS, or 3.6 % NaCl in PBS were intranasally administered with mice, and *Il6* expression normalized to *Gapdh* in nasal tissue were determined 4 h after the administration. Each symbol represents data from an individual animal. Bars indicate median. Data are representative of two separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ as determined by one-way ANOVA and Dunnett's multiple comparisons test vs naive. See also Supplementary Fig. 2.

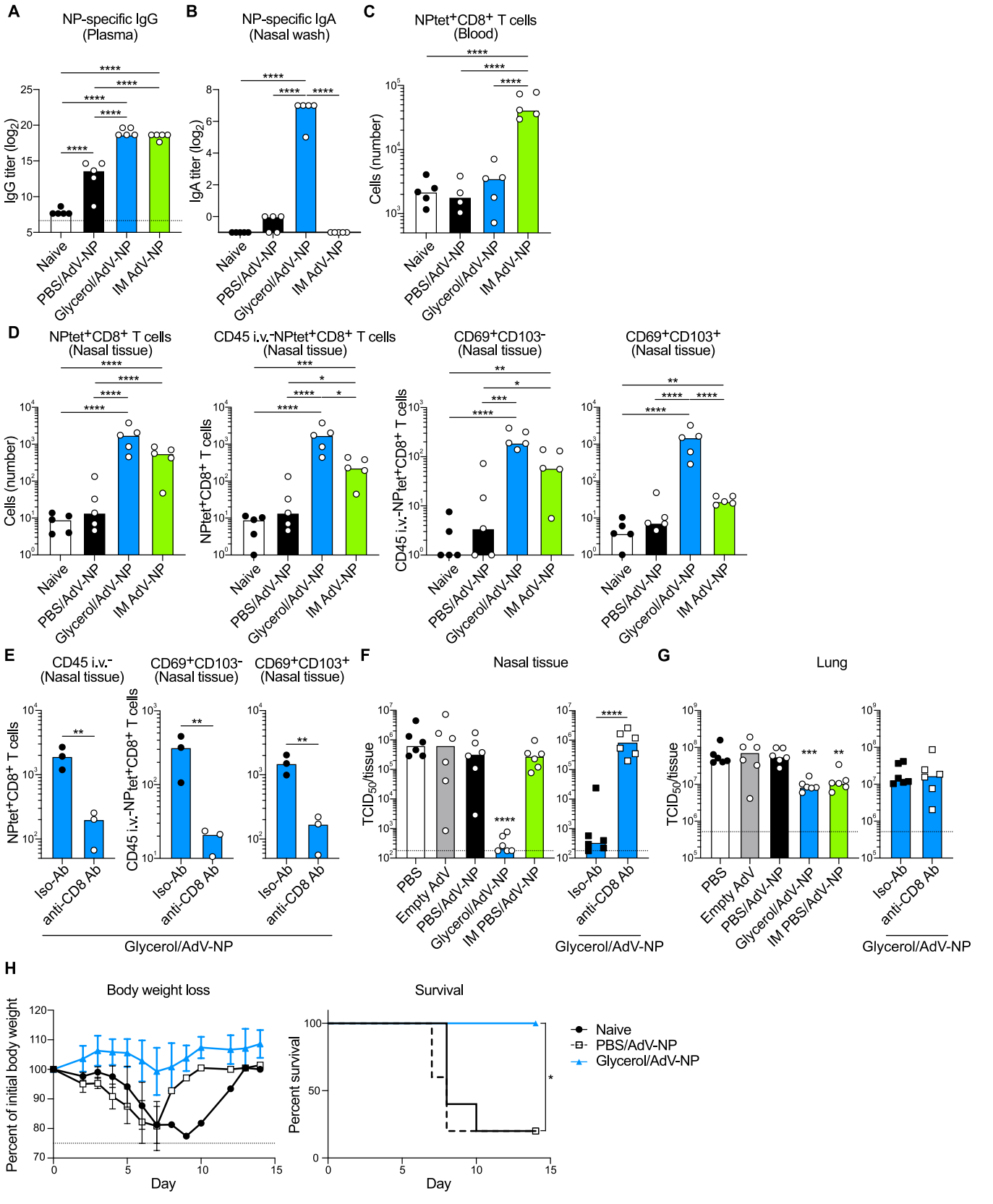

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Fig. 6. Hypertonic glycerol AdV-NP intranasal vaccine protects mice against influenza virus infection in upper and lower respiratory tracts. C57BL/6 mice were administered with AdV-NP (5×10^8 IFU/mouse) or Empty AdV in either 6 μL PBS or 10 % glycerol in PBS intranasally or in 20 μL PBS intramuscularly. (A–D) Twenty-eight days after vaccination, blood, nasal wash, and nasal tissues were harvested 5 min after the intravenous anti-CD45 antibody injection. NP-specific IgG in plasma (A) and NP-specific IgA in nasal wash (B) were measured using ELISA. (C) $CD90^+CD8^+NPtet^+$ T cells in blood were analyzed via flow cytometry. (D) $CD90^+CD8^+NPtet^+$ T cells and CD45 i.v.$^-CD90^+CD8^+NPtet^+$ T cells in nasal tissue, and expression of CD69 and CD103 on the CD45 i.v.$^-CD90^+CD8^+NPtet^+$ T cells were analyzed via flow cytometry. (E) Anti-CD8 depleting antibodies were treated 3 consecutive days from day 25 after the immunization, and nasal tissue was harvested to analyze the depletion of $CD8^+$ T cells in nasal tissue following anti-CD45 intravascular staining on day 28. (F) Twenty-eight days after the immunization with or without anti-CD8 antibody treatment, the upper respiratory tract of the mice were intranasally infected with Influenza PR8 (1200 $TCID_{50}$/mouse) in 6 μL PBS. Viral loads in nasal tissues 3 days after the infection were analyzed. (G and H) Twenty eight days after the immunization with or without anti-CD8 antibody treatment, lower respiratory tract of the mice were intranasally infected with Influenza PR8 (12 $TCID_{50}$/mouse) in 30 μL PBS. Viral loads in the lungs 5 days post-infection were analyzed (G). Body weights and survival were monitored each day after the challenge (H). Dotted lines indicate the limit of detection. Data are representative of two separate experiments. Each symbol represents data from an individual animal and each bar indicates the median (A–G), or represents group mean of n = 5 animals (H). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as determined by one-way ANOVA and Tukey's multiple comparisons test (A–D), student's *t* test (E–G), one-way ANOVA and Dunnett's multiple comparisons test vs PBS (F and G), or Kaplan-Meier curves using a log-rank test (H).

glycerol/AdV-S against D614G spike was observed; no such conflict was observed in other groups or against the delta spike of SARS-CoV-2. Thus, hypertonic AdV-S intranasal vaccine increased protective antibodies. However, both the PBS and glycerol groups exhibited reduced viral loads below the detection limit of the assay after upper respiratory infection of a murine-adapted SARS-CoV-2 strain (MA10) (Supplementary Fig. 3G). In addition, both groups completely prevented weight loss and death in mice in the lower respiratory tract infection model of MA10 (Supplementary Fig. 3H), indicating that intranasal AdV-S-induced immunity in PBS was sufficient to protect the mice in this model.

Glycerol significantly increased NP-specific IgG levels in the plasma and NP-specific IgA levels in the nasal wash of AdV-NP-intranasally immunized mice (Fig. 6A and B). Intramuscular AdV-NP induced comparable levels of NP-specific IgG compared with intranasal AdV-NP with glycerol, but did not induce NP-specific IgA in the nasal wash. Only the intramuscular AdV-NP group induced significant H-2Db $NP_{366\text{-}374}$ tetramer (NPtet)-positive NP-specific $CD8^+$ T cells in the blood compared with the naive control. Meanwhile, intranasal AdV-NP in glycerol and intramuscular AdV-NP induced NP-specific $CD8^+$ T cells in the nasal tissue, most of which were negative for intravenous CD45 staining (Fig. 6D). However, $CD69^+CD103^+$ NP-specific Trm was significantly higher in the intranasal AdV-NP in glycerol group than in the intramuscular AdV-NP group, whereas $CD69^+CD103^-$ cells did not differ significantly between the groups (Fig. 6D). Following upper respiratory challenge with PR8, we found that only intranasal AdV-NP with glycerol resulted in a near-complete reduction of viral loads in nasal tissue. This protection was canceled by 3-consecutive days pre-administration of anti-CD8 depleting antibodies[24] (Fig. 6E and F). In contrast, following a lower respiratory challenge with PR8, intranasal AdV-NP with glycerol and intramuscular AdV significantly reduced the viral loads in lungs, whereas intranasal AdV-NP in PBS had no effect. Notably, the protection provided by intranasal AdV-NP with glycerol was unaffected by pre-treatment with anti-CD8 antibodies (Fig. 6E and G). Consistent with the viral loads, intranasal AdV-NP with glycerol vaccine completely protected mice from the weight loss and deaths caused by lower respiratory PR8 infection (Fig. 6H). Collectively, the glycerol-mediated enhancement of immunity by intranasal AdV-NP vaccines is more protective than that induced by intranasal AdV-NP in PBS alone.

Glycerol increases immunogenicity of adjuvanted intranasal subunit vaccines

We tested whether hypertonic glycerol enhances AdV immunogenicity following intramuscular injection. AdV-OVA dispersed in PBS or in PBS containing 10 % glycerol was intramuscularly injected into mice, and OVA-specific IgG and $CD8^+$ T cells were evaluated 28 days after the immunization. Considering that intramuscular AdV-OVA exerts extremely strong immunogenicity, two doses of AdV were tested (i.e., 5×10^6 and 5×10^8 IFU) to evaluate the adjuvanticity of glycerol. Although we observed the increase in OVA-specific IgG responses on comparing 5×10^6 and 5×10^8 IFU of AdV-OVA, no significant differences were noted between the AdV-OVA-treated groups in both PBS and PBS containing 10 % glycerol (Fig. 7A). Additionally, OVA-specific $CD8^+$ T cells in the blood were not increased by glycerol with either AdV dosage (Fig. 7B). Thus, glycerol enhances the immunogenicity of AdV when administered intranasally, but not via intramuscular injection.

Furthermore, we evaluated the adjuvanticity of hypertonic glycerol for a protein antigen using purified OVA, followed by either intramuscular or intranasal priming and boost immunizations. Neither OVA-specific IgG in plasma nor OVA-specific IgA in nasal wash changed between the groups intramuscularly immunized with OVA without an adjuvant in PBS or in 10 % glycerol PBS; this was also true in the groups immunized intranasally (Fig. 7C). Glycerol did not increase systemic and mucosal antibody responses following intramuscular immunization with OVA supplemented with an adjuvant cyclic-di-GMP (c-di-GMP), although c-di-GMP appeared to function as an adjuvant for intramuscular immunizations as OVA-specific IgG in the plasma was increased compared with intramuscular injection with OVA alone (Fig. 7C and D). In contrast, glycerol significantly increased systemic IgG and mucosal IgA responses following intranasal immunization with OVA supplemented with adjuvant c-di-GMP (Fig. 7D). These data suggest that glycerol does not exhibit adjuvanticity and does not enhance the immunogenicity of parental vaccines but increases the immunogenicity of intranasal vaccines that already demonstrate certain immunogenicity.

To explore the mechanism of the enhanced immune responses by glycerol in adjuvanted subunit vaccines, we first evaluated the distribution of intranasally treated tdTomato as a model protein antigen (Fig. 7E and F). Although 10 % glycerol increased tdTomato uptake in nasal epithelial cells compared with that in mice treated intranasally with tdTomato in PBS, we did not observe increased uptake of tdTomato in DCs in nasal tissue. Thus, glycerol might not increase antigen uptake in DCs, which can lead to stronger immune responses. As glycerol increased the immunogenicity of OVA + c-di-GMP vaccine but not of the OVA-only vaccine, we evaluated whether glycerol enhanced adjuvanticity of intranasally administered c-di-GMP (Fig. 7G). We found that co-administration of glycerol and c-di-GMP increased *Il1a*, *Il1b*, *Il12p40*, and *Il6* expression compared with that of PBS and c-di-GMP. Thus, the enhanced adjuvanticity of c-di-GMP conferred by glycerol could be a mechanism underlying the enhanced immunogenicity of the OVA + c-di-GMP vaccine. Further, the addition of 10 % glycerol did not increase PR8 infection in nasal tissues, suggesting limited versatility to increasing the osmolality of intranasal vaccines (Supplementary Fig. 4).

Discussion

Among respiratory mucosal vaccines, intranasal vaccines administered as droppers or spray offer several advantages over pulmonary vaccines that are aerosols orally inhaled into the deep lung, including their ease of administration and less concern for patients with

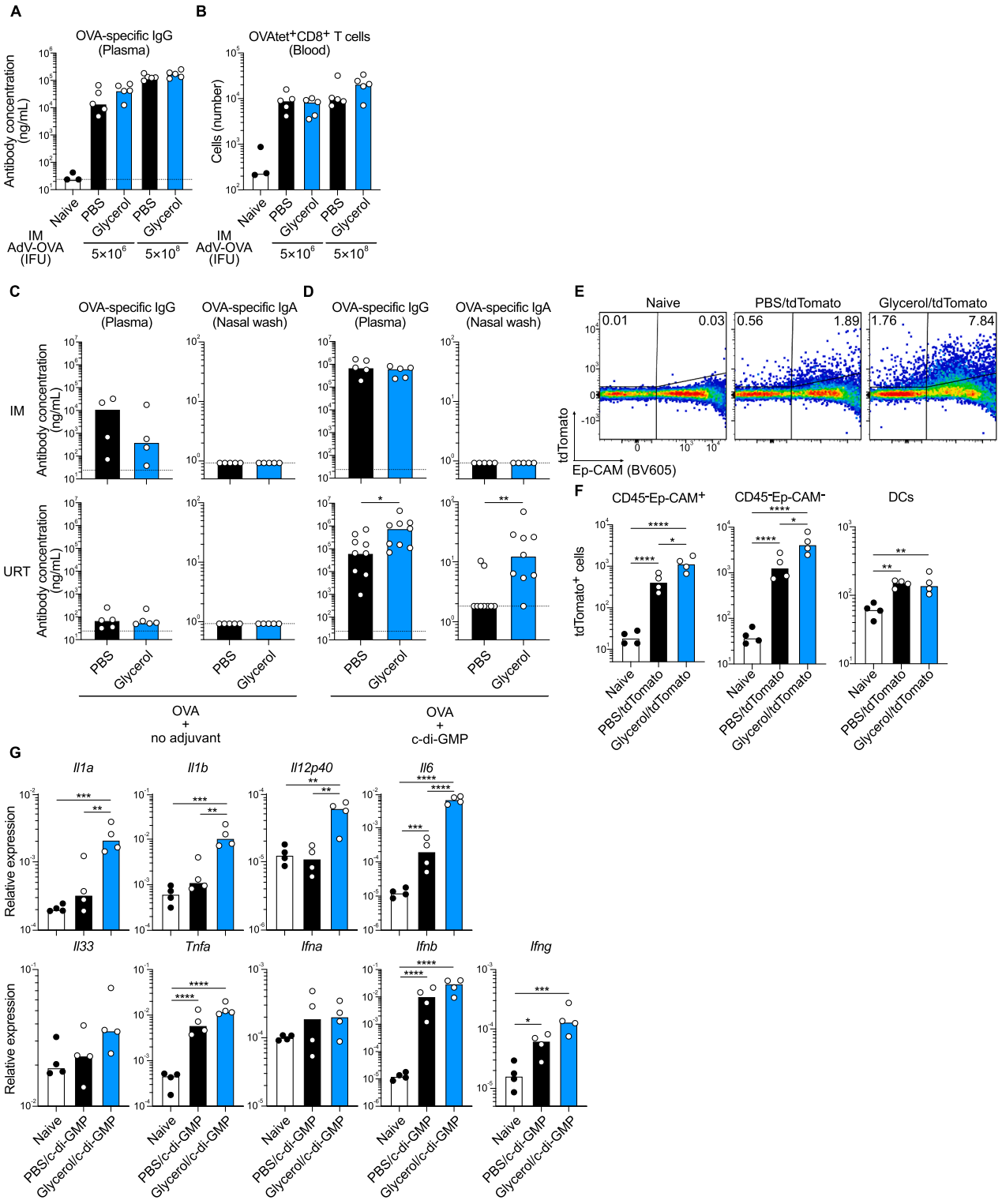

Fig. 7. Hypertonic glycerol increase immunogenicity of an intranasal subunit vaccine with adjuvant. (A and B) C57BL/6 mice were intramuscularly injected with AdV-OVA (5×10^6 or 5×10^8 IFU/mouse) in PBS or 10 % glycerol in PBS. Blood was collected after 28 days. OVA-specific IgG in plasma was determined using ELISA (A). Live $CD90^+CD8^+OVAtet^+$ T cells (blood) were enumerated by flow cytometry (B). (C and D) OVA protein (5 μg/mouse) with (C) or without (D) c-di-GMP (3 μg/mouse) were injected intramuscularly or administered intranasally on day 0 and 21. OVA-specific IgG in plasma and OVA-specific IgA in nasal wash were measured 14 days after the second immunization using ELISA. Dotted lines indicate the limit of detection. (E and F) C57BL/6 mice were intranasally treated with 6 μL tdTomato (18 μg/mouse) in PBS or 10 % glycerol. Nasal tissues were harvested for analyzing distribution of tdTomato by flow cytometry 6 h after the treatment. Representative plots of nasal tissues gated on live $CD45^-$ cells (E) and their summary data (F). (G) mRNA expression levels of inflammatory cytokine normalized to *Gapdh* in nasal tissue 4 h after the intranasal treatment of c-di-GMP (3 μg/mouse) in PBS or 10 % glycerol in PBS. Data are representative of two separate experiments. Each symbol represents an individual animal, and each bar indicates the median. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as determined by student's *t* test (A–D), or one-way ANOVA and Tukey's multiple comparisons test (F and G).

pulmonary comorbidities.[16] Thus, clinical trials are actively researching intranasal vaccines over pulmonary vaccines. However, the immunogenicity of intranasal vaccines appears to have room for improvement in many cases.[16] In this context, we found that a small volume of intranasal AdV vaccine dispersed in PBS (URT group) better recapitulated the low immunogenicity of intranasal AdV vaccine in humans compared with a large volume (TRT group) (Fig. 1).[21–23] On the contrary, the strong systemic and mucosal immunogenicity of the large-volume intranasal vaccines is more consistent with other reports that evaluated intranasal AdV vaccine immunogenicity in murine models.[17–19] Given that pulmonary AdV vaccines have shown promising immunogenicity in macaques and human,[31,32] the large-volume intranasal AdV vaccine model appears to be more representative of human pulmonary vaccines than human intranasal vaccines. Depending on whether an intranasal or pulmonary vaccine is used in humans, careful consideration of the administration volume in mouse models would result in better estimates of vaccine immunogenicity in humans.

We then demonstrated that addition of widely accepted safe additives, such as glycerol, sodium chloride, glucose, and mannitol, into intranasal AdV for hypertonic preparations increased the immunogenicity of intranasal AdV. Although these additives are commonly used in medicines administered via all kinds of routes with long proven safety, their hypertonic use in nasal vaccines might carry potential safety risks. Hypertonic saline nasal lavage has been used for many types of sinonasal diseases and has greater benefits on symptoms than does isotonic saline, with less than 5 % usage resulting in minor adverse effects. However, hypertonic saline causes the minor adverse effects more frequently than isotonic saline, such as a burning sensation and nasal irritation.[33] Considering that the sensation of heat and pain clearly depends on the degree of hypertonicity in injectable drugs,[34] hypertonic vaccines might cause pain depending on the degree of hypertonicity and volume. A previous study that evaluated pain associated with administering hypertonic saline by nasal spray in humans showed that less than three times the tonicity of saline did not induce pain, but spraying more than six times clearly induced pain.[35] In our model, two times tonicity could enhance the immunogenicity of the vaccine (Fig. 4A–C) without significantly increasing *Il6* levels (Fig. 5D), suggesting that hypertonic intranasal vaccine with optimal osmolarity might enhance the immunogenicity of vaccines without sacrificing the safety and ease of vaccine use. However, the potential discomfort experienced may be dependent on the volume and administration method (e.g. spray or droplet) of the vaccine and nose structure. Therefore, future studies are required for the optimal form in human use during vaccine development.

Stabilizing the mucus layer and PCL to form a protective mucosal barrier requires higher osmotic pressure in the PCL than in the mucus layer.[6] Thus, disrupting this balance by increasing the osmotic pressure of mucus layer causes PCL collapse, making it more susceptible to infection.[6,36,37] The normal mucus layer is isotonic[38]; therefore, hypertonic intranasal vaccines should draw water out from the mucus layer to increase its osmotic pressure. Hypertonic glycerol promoted AdV infection and antigen expression in the nasal epithelial cells (Fig. 3), presumably by promoting AdV access to the epithelial surface (Fig. 4H and I), resulting in increased *Il1b*, *Il12p40*, *Infb*, and *Ifng* levels in nasal tissues (Fig. 5C). Considering that innate immune activation accompanying AdV-infection is essential for AdV immunogenicity,[39] both the increased expression of antigen and enhanced innate immune activation could explain the enhanced antigen-specific acquired immune responses induced by hypertonic AdV intranasal vaccines.

Mucoadhesive CVP did not increase AdV infection in nasal epithelial cells (Fig. 3A) or intranasal AdV immunogenicity (Fig. 2C–F), although they showed potential to increase intranasal retention of solutes (Fig. 3E). These results suggest that unlike subunit vaccines, increasing intranasal AdV retention may not be effective in promoting immunogenicity. In contrast, hypertonic glycerol increased AdV immunogenicity, even though it promoted clearance from the nasal cavity rather than increasing recombinant luciferase retention (Fig. 3E). Considering that the mesh structure of cell-tethered mucin in PCL has been shown to effectively block AdV infection in the respiratory epithelia,[13] the major effect of hypertonic preparations might be disabling the physical barrier in PCL rather than delaying mucosylial clearance. The promoted clearance by glycerol within 4 h suggests a short window of potential PCL abnormality. In contrast, hypertonic glycerol did not increase PR8 infection in nasal tissue (Supplementary Fig. 4). As sialic acid, which is abundant on the cilia, serves as a receptor of influenza virus entry, and viruses can reach to cell body by binding to the cilia,[40,41] it is possible that PCL does not serve as an effective barrier for influenza virus, as it does for AdV. Thus, hypertonic preparations are not a universal way to increase the immunogenicity of intranasal vaccines.

AdV-NP intranasal vaccine in 10 % glycerol in PBS protected mice from upper respiratory PR8 infection, which was reversed by anti-CD8 depleting antibodies. In contrast, intramuscular AdV-NP, which induced more circulating NP-specific $CD8^+$ T cells, did not provide similar protection (Fig. 6F). These results suggest Trm, rather than circulating $CD8^+$ T cells, could provide more effective protection through hypertonic AdV-NP intranasal vaccines. In particular, $CD69^+CD103^+$ NP-specific Trm were significantly induced by the hypertonic intranasal AdV-NP than intramuscular AdV-NP, suggesting that $CD69^+CD103^+$ NP-specific Trm are responsible for conferring this protection. In contrast, the hypertonic intranasal AdV-NP and intramuscular AdV-NP provided comparable protection in the lung, which was unaffected by pre-treatment with anti-CD8 antibodies (Fig. 6G). Both vaccines induced comparable levels of NP-specific IgG in the plasma (Fig. 6A), which can reduce influenza viral titers in the lung,[42] rendering IgG-mediation of this protective capability possible. Only TRT, but not URT immunization induced $CD69^+CD103^+$ Trm in the lung (Supplementary Fig. 1). Overall, our results offer crucial insights on the importance of local vaccination for local protection. Nonetheless, future studies are required to elucidate the role of $CD69^+CD103^+$ Trm in the lung for the protection.

To increase the immunogenicity of intranasal subunit vaccines, promoting antigen delivery to APCs, potentially by increasing antigen absorption through the mucosal barrier, or APCs activation either directly or indirectly by adjuvants is required. Intranasal 10 % glycerol in PBS promoted antigen uptake in epithelial cells, but not in DCs, in nasal tissues (Fig. 7E and F). There were no apparent signs of innate immune activation as adjuvants by 10 % glycerol in PBS except *Il6* induction, though the *Il6* was not increased by 2.5 % glycerol in PBS, which enhanced the immunogenicity of AdV as well as 10 % (Fig. 5). Furthermore, 10 % glycerol did not increase the immunogenicity of either intramuscular AdV, intramuscular OVA with/without adjuvant, or intranasal OVA without adjuvant (Fig. 7A–C). These results suggest that hypertonic glycerol does not promote antigen delivery to APCs or possess immune-stimulating properties as adjuvants. In contrast, hypertonic glycerol increased intranasal OVA immunogenicity with c-di-GMP vaccine (Fig. 7D). Considering that hypertonic glycerol enhances epithelial access of solutes (Fig. 4H and I), it is plausible that OVA and c-di-GMP, which can function as an adjuvant in epithelial cells, have better access to epithelial cells in hypertonic preparations.[43] In addition, we observed the enhanced c-di-GMP-mediated innate immune activation by hypertonic glycerol (Fig. 7G). This suggests that increasing c-di-GMP delivery to epithelial cells to exert increased adjuvanticity might be the mechanism underlying the improved antibody responses to adjuvanted subunit intranasal vaccines mediated by hypertonic preparations.

The intranasal absorption of peptide or protein formulations in circulating blood can be modified by their osmolarity.[44,45] In addition, plasmid DNA transfer to respiratory epithelia has been reported to be enhanced by hypotonic preparations and not by hypertonic.[46] These reports prompt us to consider the influence of osmotic pressure of intranasal vaccine preparations on vaccine efficacy. However, the relationship between osmotic pressure and intranasal vaccine immunogenicity has not been clarified. In this study, we revealed that this basic

parameter of intranasal vaccine formulations can significantly affect vaccine efficacy. Future studies should investigate whether this concept can be applied to other mucosal vaccines in addition to intranasal vaccines.

One important limitation of our study is that the osmotic pressure—more than twice that of isotonic pressure—that increased intranasal AdV immunogenicity in our mouse model (Fig. 4B–G) may not be applicable in other animal models or humans. Considering the mechanism by which hypertonic preparations of intranasal vaccines increase immunogenicity, the osmotic pressure required to increase the immunogenicity of intranasal vaccines may depend on the contact area between the mucosal surface and vaccines and the volume of vaccine drop on the contacted area. Therefore, several factors, including the administration volume, administration method (spray or droppers, if sprayed, how big the spraying vaccine droplet is), and volume and area of the nasal cavity, affect osmotic pressure to increase intranasal vaccine immunogenicity. In addition, certain proteins may not be stable in solutions with high ion intensity. Thus, using salts to increase osmolality entails particular attention for vaccine stability. Every formulation of an intranasal vaccine may eventually require examination to determine its effective value of osmotic pressure. Nevertheless, simple method for improving the immunogenicity of intranasal vaccines would be of immediate relevance for clinical development.

Methods

Mice

Female C57BL/6J and BALB/c mice (aged 6–7 weeks) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). They were housed in a room with a 12-h light/dark cycle with unrestricted access to food and water. All animal experiments were performed in accordance with the Osaka University's Institutional Guidelines for the Ethical Treatment of Animals and were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan (BIKEN-AP-R02-14-5).

AdV

The cDNA sequences encoding OVA, S from SARS-CoV-2 (Wuhan-Hu-1) with a glycine substitution at 614 (D614G) and GSAS substitution at the furin cleavage site (R682G, R683S, R685S), NP from H1N1 influenza A virus strain A/Puerto Rico/8/34 (PR8), or enhanced GFP were cloned into the pcDNA3.1 expression plasmid (Thermo Fisher Scientific, Waltham, MA, USA, #V79020). The expression cassettes, including the CMV promoter and bGH poly A signal, were then cloned into the plasmid Adeno-X™ Adenoviral System 3 (Takara Bio, Shiga, Japan, #632269) (E1/E3 deleted, serotype 5). PacI-digested plasmid AdV-OVA, AdV-S, AdV-NP or AdV-GFP were transfected into HEK293 cells (JCRB Cell Bank, Osaka, Japan, #JCRB9068) using Lipofectamine 2000 (Thermo Fisher Scientific, #11668019). The resulting AdV was further amplified in HEK293 cells and purified by cesium chloride gradient ultracentrifugation twice, dialyzed against 0.01 M Tris-HCL (pH 7.5) buffer containing 10 % glycerol (not in AdV-NP) and 0.001 M $MgCl_2$,and stored at −80 °C, after quantifying the number of virus particles through absorbance at 260 nm.[47] IFUs were measured using the Adeno-X Rapid Titer Kit (Takara Clontech, Shiga, Japan, #Z2250N) following the manufacturer's instructions. The stock concentrations of AdV-OVA, AdV-S, AdV-NP, empty AdV and AdV-GFP were 10.3×10^{11}, 4.74×10^{11}, 2.42×10^{10}, 3.85×10^{11} and 4.57×10^{11} IFU/mL, with particle-to-infectivity ratios of 6.4, 7.3, 10.1, 6.2 and 8.0, respectively. Experiments using AdV were approved by the Institutional Review Board of the Research Institute for Microbial Diseases, Osaka University (protocol number: BIKEN-00181-005).

Recombinant protein

Low-endotoxin OVA for immunization was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan, #015-24731). OVA for ELISA and luciferase assays was procured from Sigma-Aldrich (St. Louis, MO, USA, #A2512 and #SRE0045-1MG, respectively). NP proteins from H1N1 influenza A virus strain PR8 were purchased from Sino Biological (Beijing, China, #11675-V08B). S protein from SARS-CoV-2 (Wuhan-Hu-1) and tdTomato was prepared in-house. Briefly, the cDNA of S ectodomain (amino acids 1-1208), which has a glycine substitution at 614 (D614G), proline substitutions at 986 and 987 (K986P, V987P), and a GSAS substitution at the furin cleavage site (R682G, R683S, R685S), with the bacteriophage T4 fibritin foldon sequence (GYIPEAPRDGQAYVRKDGEWVLLSTFL) followed by an octahistidine tag at C-terminal or tdTomato (Addgene, #182340) with human IgGκ signal peptide followed by an hexahistidine tag at N-terminal, was cloned into the pcDNA3.1 expression plasmid (Thermo Fisher Scientific). The expression vectors were transfected into Expi293F cells according to the manufacturer's instructions (Thermo Fisher Scientific, #A14525). Four days after transfection, the cell culture supernatant was centrifuged and the S protein-containing supernatants were harvested. Subsequently, S protein was purified using AKTA Explorer chromatography system with a Ni-Sepharose HisTrap FF column (GE Healthcare, Chicago, IL, USA #17531901) and Superose 6 Increase 10/300 GL column (GE Healthcare, #29091596).

Reagents

Pork skin-derived gelatin hydrolysate was purchased from Nitta Gelatin Inc. (Osaka, Japan, #633-44311). Glycerol, methylcellulose #15, sodium chloride, and D(+)-Glucose were acquired from Nacalai Tesque (Kyoto, Japan, #17017-93, #11671-22, #31320-05, and #16806-25, respectively). Sodium alginate 80-120 and D(−)-mannitol were procured from FUJIFILM Wako Pure Chemical (#194-13321 and #139-00842, respectively). CVP dissolved in PBS was a kind gift from Toko Yakuhin Kogyo Co. Ltd. (Toyama, Japan).

Vaccination

For most intranasal immunizations (URT) of AdV or OVA (5 μg/mouse/immunization) with or without c-di-GMP (3 μg/mouse/immunization) except TRT immunization, mice were intranasally administered vaccine solutions of 6 μL (3 μL/nostril). AdV for URT was prepared in water or PBS and administered at 1×10^8 or 5×10^8 IFU dose per mouse. In some experiments, glycerol, methylcellulose, sodium alginate, CVP, sodium chloride, D(+)-glucose, or D(−)-mannitol were added to the vaccine solutions at final concentrations as described in figure or legends (w/v%). The carryover of glycerol from AdV stock was 0.16–1.8 % in the final vaccine solution, which was not counted in the final glycerol concentrations in glycerol-added groups. Mice were intranasally administered with 30 μL (15 μL/nostril) solution containing 5×10^8 IFU of AdV-OVA in PBS in TRT. Mice were intramuscularly injected with 5×10^6 or 5×10^8 IFU of Ad-OVA in left tibialis anterior muscle in 20 μL of PBS or 10 % glycerol in PBS. All vaccinations were performed under anesthesia induced by an intraperitoneal injection of a mixture of midazolam (4 mg/kg) (Maruishi Pharmaceutical Co. Ltd. Osaka, Japan), butorphanol tartrate (5 mg/kg) (Meiji, Tokyo, Japan), and medetomidine hydrochloride (0.3 mg/kg) (ZENOAQ, Fukushima, Japan). The mice were awakened from anesthesia using atipamezole hydrochloride (ZENOAQ) after vaccination.

Sample collection

Nasal washes were collected as follows. The lower jaw of each mouse was removed to expose the pharynx to the nasal cavity. A pipette tip was then inserted into the pharynx, and the nasal cavity was flushed with

200 μL of PBS. PBS was collected in a 1.5 mL tube. The supernatant was collected after centrifugation at 5000 × *g* for 20 min at 4 °C as the nasal wash. BALF was collected by injecting 1 mL of PBS into the lungs through trachea using a cannula (TERUMO, Tokyo, Japan, #SR-FF2225), and PBS was collected after 10 s. The BALF was the supernatant of the PBS collected after centrifugation at 5000 × *g* for 20 min at 4 °C.

ELISA

ELISA plates (Corning, NY, USA, #3690) were coated overnight with antigen (OVA: 10 μg/mL PBS; S, NP: 1 μg/mL carbonate buffer except NP: 10 μg/mL carbonate buffer for NP-specific IgA) at 4 °C. The plates were washed with PBS-T (PBS containing 0.05 % Tween-20) three times, with subsequent washing performed immediately before every procedure. The coated plates were then incubated with blocking solution (1 % Block Ace, KAC, Kyoto, Japan, #UKB80) for 1 h at room temperature. Sample dilutions were added to the plates and incubated for 2 h at room temperature, followed by 1-h incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or IgA (Southern Biotech, Birmingham, AL, USA #1030-05, #1040-05) at room temperature. The color reaction was developed using tetramethylbenzidine (Nacalai Tesque, #05299-54) and stopped using 2 N H_2SO_4. Absorbance (OD) at 450–570 nm–was measured using a microplate reader (Power Wave HT, BioTek, Winooski, VT, USA). The concentration of antigen-binding antibodies was determined with reference to standard curves using monoclonal antibodies against the antigen (OVA-specific IgG: BioLegend San Diego, CA, USA #520501; OVA specific IgA: Chondrex Inc. Woodinville, WA, USA #7090; spike-specific IgG: R&D Systems, Minneapolis, MN, USA #MAB105808-SP). Antibody titer was determined as the end-point dilution when 0.1 $OD_{450\text{-}570}$ > background.

Intravascular staining

To distinguish cells in vasculature and tissue parenchyma for flow cytometry, mice were intravenously injected 3 μg of PE anti-CD45 (clone: 30-F11; BioLegend, #103106) in 300 μL of PBS, 5 min before euthanizing mice with CO_2 and harvesting tissues.

Preparation of single cell suspension from nasal tissue

Nasal tissues were harvested by removing the head and dissecting the lower jaw, tongue, and connective tissues to expose the soft palate of the upper jaw. Then, the skull was vertically cut and the tissues and small bones were scraped out from both sides of the nasal passages. The front incisors were removed to reveal the anterior end of the soft palate, which was then peeled back to remove NALTs. The remaining tissue was the nasal tissue; after cutting it into half along the nasal septum, the nasal cavity and turbinate were scraped out from the bone using a Volkmann bone curette (Takasago Medical Industry Co. Ltd., Tokyo, Japan, #TKZ-F1220-0506). To analyze CD8 T cells, a single cell suspension of nasal tissue was obtained by passing it through a 70 μm cell strainer (Falcon, #352350) after further mechanical disruption. For epithelial cells analysis, the tissues were digested in 1 mL TrypLE Express (Thermo Fisher Scientific, #12605010) with DNase I (100 U/mL; Wako, #047-26773) in a shaking incubator at 200 rpm for 45 min at 37 °C before passing through the strainer. The resulting cells were treated with red cell lysis buffer.

Preparation of single cell suspension from lungs

The lungs were minced and digested with collagenase IV (200 U/mL; Gibco, #17104-019) and DNase I in RPMI1640 supplemented with 5 % FBS and 20 mM HEPES in a shaking incubator at 200 rpm for 1 h at 37 °C. After incubation, 5 mL of RPMI1640 medium was added and the mixture were processed using a gentleMACS Dissociator (Miltenyi Biotec, North Rhine-Westphalia, Germany, #130-096-334). The resulting cells were filtered through a 70 mm cell strainer and treated with red cell lysis buffer.

Flow cytometry

Single cell suspensions were blocked with anti-mouse CD16/CD32 antibody (clone: 93, BioLegend, San Diego, CA, USA, #101319) and stained with fixable viability dye eFluor 780 (Thermo Fisher Scientific), fluorescent dye conjugated antibodies and tetramers (H-2 $K^b/OVA_{257\text{-}264}$, H-2 $K^b/S_{539\text{-}546}$, H-2$K^d/NP_{366\text{-}374}$) (biotinylated monomer was obtained from NIH Tetramer Core Facility and tetramized with APC-streptavidin; Agilent Technologies, Santa Clara, CA, USA, #PJ27S-1), for 15 min at 37 °C. For intracellular staining of GFP, the cells were fixed with Fixation/Permeabilization (BD Bioscience, Franklyn Lakes, NJ, USA, #554714) for 20 min at 4 °C in dark. Post-incubation, cells were washed twice by BD Perm/Wash buffer (BD Bioscience, #554714) and stained with antibody against GFP at 4 °C overnight. We used the following antibodies for staining: AlexaFluor488 or AlexaFluor700 anti-mouse CD90.2 (1:400 dilution; clone:30-H12; BioLegend, #105315 or 105320, respectively), FITC anti-mouse TCRb (1:200 dilution; clone: H57-597; BioLegend, # 109205), AlexaFluor488 anti-GFP (1:2000 dilution; Thermo Fisher Scientific, #A21311), AlexaFluor555 goat anti-rat IgG (minimal x-reactivity) antibody (1:400 dilution; BioLegend, #405420), AlexaFluor647 anti-mouse CD31 (1:200 dilution; clone: 390; BioLegend, #102415), AlexaFluor647 goat anti-rat IgG (minimal x-reactivity) (1:400 dilution; clone: Poly 4054, BioLegend, #405416), Brilliant Violet 510 anti-mouse CD44 (1:200 dilution; clone: IM7; BioLegend, #103043), Brilliant Violet 605 anti-mouse CD8a (1:200 dilution; clone: 53–6.7; BioLegend, #100743), Brilliant Violet 605 anti-mouse CD326 (Ep-CAM) (1:100 dilution; clone: G8.8; BioLegend, #118227), PE anti-mouse CD45 (1:200 dilution; clone: 30-F11; BioLegend, #103105), PE-Cy7 anti-mouse CD69 (1:200 dilution; clone: H1.2F3; BioLegend, #104511), Brilliant Violet 421 anti-mouse CD103 (1:200 dilution; clone: 2E7; BioLegend, #121422), Brilliant Violet 421 anti-mouse CD45 (1:200 dilution; clone: 30-F11; BioLegend, #103134), AlexaFluor700 anti-mouse MHCII (1:200 dilution; clone: M5/114.15.2; BioLegend, # 107622), PE anti-mouse CD3 (1:200 dilution; clone: 17A2; BioLegend, # 100206), AlexaFluor488 anti-Ly6G (1:200 dilution; clone: 1A8; BioLegend, # 127626), APC anti-mouse siglecF (1:200 dilution; clone: ES22-10D8; Miltenyi Biotec, # 130-123-816), Brilliant Violet 510 anti-mouse CD11b (1:200 dilution; clone: M1/70; BioLegend, # 101263), PE-dazzle 594 anti-mouse CD11c (1:200 dilution; clone: N418; BioLegend, # 107346), and PE-Cy7 anti-mouse CD19 (1:200 dilution; clone: 6D5; BioLegend, # 115520). Neutrophils, eosinophils, monocytes, macrophages, DCs, and T cells were gated as live $CD45^+$ $MHCII^-CD3^-CD11b^+Ly\text{-}6G^+$, $CD45^+MHCII^-CD3^-CD11b^+Ly\text{-}6G^-siglecF^+$, $CD45^+MHCII^-CD3^-CD11b^+Ly\text{-}6G^-siglecF^-$, $CD45^+MHCII^+CD3^-CD11c^+$ $CD19^-$, $CD45^+MHCII^+CD3^-CD11c^+CD19^-$, and $CD45^+MHCII^+CD3^+$, respectively. Flow cytometry was performed using the Attune NxT Flow Cytometer (Thermo Fisher Scientific). Flowjo software (TreeStar, Woodburn, OR, USA) was used for the analysis.

Histologic analysis

Twenty four hours after the intranasal administration of PBS, 10 % glycerol in PBS, or 30 % glycerol in PBS with or without AdV-OVA (5×10^8 IFU/mouse) (6 or 30 μL/mouse, as indicated in figures), the nasal tissues were collected and fixed in 4 % paraformaldehyde in PBS for 24 h at room temperature. The fixed tissues were washed with PBS, embedded in paraffin, and sectioned. The tissue sections were then stained with hematoxylin and eosin for histologic analysis. The process of sectioning and evaluating the slides for pathological findings was performed by the Applied Medical Research Laboratory (Osaka, Japan) under blinded conditions regarding the treatment.

Neutralization assay

VeroE6/TMPRSS2 was seeded at 1.2×10^4 cells per well on 96-well half-white plates (Greiner BIO-ONE) and incubated for 24 h. Serum samples were heat-inactivated for 30 min at 56 °C and 4-fold serial dilutions of serum or nasal wash were mixed 1:1 with a replication-deficient VSV-based pseudotyped viruses expressing the spike protein from SARSCoV-2, and incubated for 1 h at 37 °C. The mixture of serum and the pseudotyped viruses were then added to the wells and incubated for 48 h. Equal volume of ONE-Glo-EX Reagent (Promega) to the volume of the culture medium was added and luminescence was measured using a GloMax Discover Microplate Reader (Promega, #GM3000).

Virus challenge

BALB/c mice were intranasally administered 5×10^4 PFU of MA10 (mouse-adapted strain of SARS-CoV-2 generated from SARS-CoV-2 NIID strain using CPER method)[48,49] in 5 μL (2.5 μL/nostril) of PBS for upper respiratory infection and 2×10^5 PFU of MA 20 μL of PBS (10 μL/nostril) for lower respiratory infection under anesthesia. Experiments with live SARS-CoV-2 were performed within a biosafety level 3 facility at Osaka University, adhering to stringent guidelines. C57BL/6J mice were intranasally administered with 1200 median tissue culture infectious dose (TCID50) of Influenza A/Puerto Rico/8/34 (PR8, H1N1) in a total of 6 μL (3 μL/nostril) of PBS for upper respiratory infection and 12 TCID50 of PR8 in a total of 30 μL (15 μL/nostril) of PBS for lower respiratory infection under anesthetics. Body weights were measured just before viral lower respiratory infection (day 0) and then on following days. The percent weight change was calculated as the weight on a certain day divided by the weight on the day of infection (day 0). The survival of the mice was monitored daily after infection. To measure the viral loads of MA10 in nasal tissue after upper respiratory infection, the nasal turbinates were homogenized, and the supernatant was collected after centrifugation. The supernatant was titrated for measuring PFU in VeroE6/TMPRSS2. To measure the viral loads of PR8 in upper or lower respiratory tract, total RNA was extracted from the nasal cavity or lung tissues using the TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by ReverTra Ace qPCR RT Master Mix with a gDNA Remover (Toyobo) and used for qPCR with SYBR™ Green qPCR Master Mix (Thermo Fisher Scientific) (Light Cycler 480 SYBR green I Master, Roche). Primers specific for Np (forward, 5′-GCCATAAGGA CCAGGAGTGG-3′; reverse, 5′-GCTGAATGCTGCCATAACGG-3′) were used. The viral loads were determined by a standard curve from RNA extracted from a stock virus with a known TCID50 using the High Pure Viral RNA kit (Roche). Experiments using viruses were approved by the Institutional Review Board of the Research Institute for Microbial Diseases, Osaka University (protocol numbers: BIKEN-00006-010 and BIKEN-00137-052).

Quantifying AdV infection in nasal tissue

Twenty-four hours after the intranasal administration of PBS, 10 % glycerol in PBS, or 0.55 % CVP with AdV-OVA (5×10^8 IFU/mouse) in 6 μL, the nasal tissues were harvested, and DNA was purified with the DNeasy Blood&Tissue kit (Qiagen, Hilden, Germany). qPCR was performed with SYBR™ Green qPCR Master Mix (Thermo Fisher Scientific) in LightCycler 480 II (Roche). Primers specific for OVA were used (forward, 5′-ATGTCCTTCAGCCAAGCTCC-3′; reverse, 5′-TCA-GAAGCCATTGATGCCACT-3′). AdV genomic copies were determined by a standard curve derived from DNA extracted from a stock AdV-OVA.

Immunofluorescence of nasal tissue

The nasal tissues were extracted as described in the single cell suspension preparation except the NALT removal. They were then fixed in 30 mL of 4 % paraformaldehyde for 6 h at 4 °C. After washing with PBS, the nasal tissues were decalcified in 40 mL of 20 % EDTA (EDTA2Na 45 g + EDTA4Na 50 g/500 mL dry weight) for 48 h at room temperature. The EDTA solutions were exchanged after 24 h. The nasal tissues were then embedded in OCT compound (Sakura Finetek, Tokyo, Japan, #4583) and sored until sectioning at −80 °C. Nasal tissues were sectioned into 10 μm using Cryostat (Thermo Fisher Scientific, #HM525NX). Sections on glass slide glass were re-fixed with 4 % paraformaldehyde for 15 min at room temperature. After rinsing twice with PBS, sections were incubated with blocking buffer (2 % BSA, 2 % rabbit serum, 2 % goat serum in PBS supplemented 0.1 % tween-20) for 1 h at room temperature. The sections were then washed three times with PBS containing 0.5 % BSA and 0.1 % tween 20 (wash buffer) and incubated with anti-GFP AlexaFluor488 (1:2000 dilution, Thermo Fisher Scientific, #A21311) and anti-α-tubulin AlexaFluor647 (1:2000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA, # SC-23950 AF647) in wash buffer. After 1-h incubation at room temperature followed by three washes, the slides were mounted with a mounting medium containing DAPI (Invitrogen, #P36981). All staining was performed in the dark to protect sections from light. The images were acquired using an Olympus VS-200 research slide scanner (Olympus, Tokyo, Japan).

Intranasal retention of recombinant luciferase

Mice were intranasally administered with 1×10^6 units of recombinant luciferase (Sigma-Aldrich, St. Louis, MO, USA, #SRE0045-1MG) in a total of 6 μL (3 μL/nostril). Mice were euthanized 1, 4, or 8 h after administration, and nasal washes were collected as described above. The nasal washes were transferred to 96-well white plate (Greiner Bio-One, Kremsmuster, Australia, #675083), followed by the addition of 30 μL luciferin (Promega, Madison, WI, USA, #E605A). The relative luminescence unit (RLU) was measured using a GloMax Discover Microplate Reader (Promega, #GM3000).

Intranasal anti-Ep-CAM antibody administration

Mice were intranasally administrated with 0.1 μg of anti-Ep-CAM antibody (BioLegend, #118201) in 6 μL (3 μL/nostril). After 2 h, nasal tissues were harvested after washing the nasal cavity five times with 200 μL of PBS in the same manner the nasal washes were collected.

Intranasal absorption assay

Mice were intranasally treated with 1.25 mg FITC-dextran with a mean molecular weight of 150,000 (FD150: TdB Labs, Uppsala, Sweden) in 6 μL of PBS, 10 % glycerol in PBS, or 1 % Capric acid (Tokyo Chemical Industry Co., Tokyo Japan) in PBS. One hour post-treatment, blood was collected through cardiocentesis and centrifuged 5000 × *g* at 4 °C. The FITC fluorescence was measured from the resulting plasma, which was diluted 1:5 with PBS, using a GloMax Discover Microplate Reader in a 96-well black plate.

Inflammatory cytokines mRNA expression

Mice were intranasally treated 6 μL of various solution described in figure legends. Four or twenty four hour after the administration, nasal total RNA were extracted by washing the nasal cavity with 400 μL TRIzol. cDNA was synthesized by ReverTra Ace qPCR RT Master Mix with a gDNA Remover (Toyobo) and used for qPCR with SYBR™ Green qPCR Master Mix (Thermo Fisher Scientific) (Light Cycler 480 SYBR green I Master, Roche). Primers specific for *Il1a* (forward, 5′-CGTGTTGCTGAAGGAGTTGC-3′; reverse, 5′-TCTGGATAAGCAGCTG ATGTGA-3′), *Il1b* (forward, 5′-TCTTTGAAGTTGACGGACCC-3′; reverse, 5′-TGAGTGATACTGCCTGCCTG-3′), *Il12p40* (forward, 5′-ATCAT-CAAACCAGACCCGCC-3′; reverse, 5′-GAGGAACGCACCTTTCTGGTT-3′), *Il6* (forward, 5′-CTGTAGCTCATTCTGCTCTGGA-3′; reverse, 5′-

CAACTGGATGGAAGTCTCTTGC-3′), *Il33* (forward, 5′-CCTGCAAGTCAATCAGGCGA-3′; reverse, 5′-ACGGAGTAGTCCTTGTCGTTG-3′), *Tnfa* (forward, 5′- CCTCTCATGCACCACCATCAA-3′; reverse, 5′- TTCTGAGACAGAGGCAACCTG-3′), *Ifna* (forward, 5′- GGACTTTGGATTCCCGCAGGAGAAG-3′; reverse, 5′- GCTGCATCAGACAGCCTTGCAGGTC-3′), *Ifnb* (forward, 5′- AACCTCACCTACAGGGCGGACTTCA-3′; reverse, 5′- TCCC ACGTCAATCTTTCCTCTTGCTTT-3′), *Ifng* (forward, 5′- GGATGCATTCATGAGTATTGC-3′; reverse, 5′-CCTTTTCCGCTTCCTGAGG-3′), *Gapdh* (forward, 5′-CAGGTTGTCTCCTGCGACTT-3′; reverse, 5′-AGCCGTATTCATTGTCATACCAGG-3′) were used.

CD8$^+$ T cells depletion

Next, 200 μg of anti-mouse CD8α (Clone: L3, Selleck Biotechnology, Kanagawa, Japan) were intraperitoneally injected 3 consecutive days[24] from day 25 after the immunization.

Distribution analysis of intranasally treated protein antigen

Mice were intranasally treated with 18 μg tdTomato in 6 μL of PBS or 10 % glycerol in PBS. Six hours after the treatment, nasal tissues were harvested for flowcytometry.

Statistical analyses

Groups were compared using Prism software (GraphPad Version 10, San Diego, CA, USA) with a two-tailed unpaired Student's *t*-test to compare two groups or one-way ANOVA or two-way ANOVA and Dunnett's test or Tukey's multiple comparisons test to compare more than two groups. Data presented in graphs with logarithmic axes were analyzed after logarithmic transformation. $P < 0.05$ was considered significant.

CRediT authorship contribution statement

Soichiro Hashimoto: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Toshiro Hirai:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Koki Ueda:** Writing – review & editing, Investigation. **Mako Kakihara:** Writing – review & editing, Investigation. **Nagisa Tokunoh:** Writing – review & editing, Investigation. **Chikako Ono:** Writing – review & editing, Methodology. **Yoshiharu Matsuura:** Writing – review & editing, Methodology. **Kazuo Takayama:** Writing – review & editing, Methodology. **Yasuo Yoshioka:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

Toshiro Hirai and Yasuo Yoshioka filed a patent application related to the content of the manuscript (JP2023-148013 and JP2024-055279). Nagisa Tokunoh and Yasuo Yoshioka are employees of The Reseatch Foundation for Microbial Diseases of Osaka University. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mucimm.2025.03.006.

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