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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
BAL, bronchoalveolar lavages; B-NP, the influenza B virus nucleoprotein; EP, electroporation; i.m, intramuscular; NP, nucleoprotein; rAd/mock, replication-defective adenovirus that expresses no Ag; RT, room temperature; T_{EM}, effector memory T cell; T_{RM}, resident memory T cell.

A "Prime and Deploy" Strategy for Universal Influenza Vaccine Targeting Nucleoprotein Induces Lung-Resident Memory CD8 T cells

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ABSTRACT

Lung-resident memory T cells (T_{RM}) play an essential role in protecting against pulmonary virus infection. Parenteral administration of DNA vaccine is generally not sufficient to induce lung CD8 T_{RM} cells. This study investigates whether intramuscularly administered DNA vaccine expressing the nucleoprotein (NP) induces lung T_{RM} cells and protects against the influenza B virus. The results show that DNA vaccination poorly generates lung T_{RM} cells and massive secondary effector CD8 T cells entering the lungs after challenge infection do not offer sufficient protection. Nonetheless, intranasal administration of non-replicating adenovirus vector expressing no Ag following priming DNA vaccination deploys NP-specific CD8 T_{RM} cells in the lungs, which subsequently offers complete protection. This novel 'prime and deploy' strategy could be a promising regimen for a universal influenza vaccine targeting the conserved NP Ag.

Keywords: Lung CD8 T_{RM}; DNA vaccine; Adenovirus vector; Intranasal; Influenza B virus

INTRODUCTION

Memory T cells established by an infection or vaccination mediate faster and stronger secondary responses to subsequent Ag encounters. Recently identified tissue-resident memory T cells (T_{RM}), which are distinct from circulating effector memory T cells (T_{EM}), reside within non-lymphoid tissues and play an essential role in controlling infections at the local tissue barriers. CD8 T_{RM} cells in the lungs protect against respiratory virus infections, as they respond immediately against incoming respiratory pathogens (1-3). However, it remains unclear which conditions efficiently induce T_{RM} formation in the airways and lungs.

DNA vaccine, a relatively new approach to vaccination, induces a wide range of immune responses. DNA vaccines encoding target Ags from various pathogens, such as influenza, hepatitis B virus, malaria, and HIV, have been widely studied in diverse disease animal models (4-7). DNA vaccination can induce CD8 T-cell immunity, but it is unknown whether parenteral administration of DNA vaccine establishes CD8 T_{RM} responses in specific tissues, such as the lungs. Parenteral administration of the DNA vaccine expressing nucleoprotein (NP) reportedly does not protect against influenza A infection, which suggests that the intramuscularly administered DNA vaccine has a limited ability to induce protective T_{RM} responses in the lungs (5).

Generating CD8 T_{RM} cells in the lungs could enhance the effectiveness of parenteral DNA vaccination. The recently introduced 'prime and pull' strategy employs systemic immunization followed by induction of local inflammation with cognate Ag, thereby attracting systemically circulating effector T cells to specific local tissues (1,8-11). Previous studies have shown that mucosal pulling of Ag-specific T cells and establishing T_{RM} cells requires an Ag encounter in the pulmonary tissue (9-12). However, it is still unclear whether local inflammation without Ag exposure is sufficient to recruit and form a CD8 T_{RM} response, which may be a more versatile strategy.

In this study, we investigated whether 'primed' Ag-specific CD8 T cells could be 'deployed' by local inflammatory signals in the absence of a cognate Ag encounter. Non-replicating adenovirus vectors are capable of triggering innate immune responses, which may potentiate vaccination (13,14). We primed influenza B NP-specific CD8 T cells by DNA vaccination and provided Ag-independent local inflammation by intranasal administration of a replication-defective adenovirus that expresses no Ag (rAd/mock). We then examined the establishment of airway and lung-resident CD8 T_{RM} cells and the protective efficacy of a 'prime and deploy' vaccine strategy.

MATERIALS AND METHODS

Mice and ethics statement

We purchased 6 to 7-week-old BALB/cAnNCriljOri mice from Orient Bio Inc. (Seoul, Korea) and maintained them in a specific pathogen-free facility. All animal studies were approved by the Institutional Animal Care and Use Committees of Ewha Woman's University (approval No. 18-005) and performed in strict accordance with the animal handling policies mandated by the Institute of Laboratory Animal Resources Guide.

Generation and validation of recombinant plasmids expressing the influenza B virus NP (B-NP)

NP gene cDNA from the B/Yamagata/16/88 virus (15) was generated by PCR using a forward primer that included a NheI restriction enzyme site and Kozak sequence to enhance translation (5′-CGGCTAGCGCCACCATGTCCAACATGGATATT-3′) and a reverse primer (5′-GCTCGAGTCATTAATAGTCGAGGTCATC-3′) that included 2 stop codons and an XhoI restriction enzyme site. We cloned DNA from PCR reactions into the pGX27 vector (16) through NheI/XhoI double digestion, resulting in pGX27/B-NP. To validate immunogen expression, HEK293 cells were transfected with 8 μg of pGX27/B-NP or pGX27 with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), used according to the manufacturer's directions. As described previously, rAd/B-NP was a positive control (15). The transfected cells were harvested after 48 h, and the resulting cell lysate and supernatant were subjected to SDS-PAGE and Western blotting using a mouse polyclonal B/Yamagata-specific antiserum and HRP—conjugated rabbit anti-mouse IgG (Abcam, Cambridge, UK) as a secondary Ab.

Vaccination

All plasmid DNA vaccines (pGX27/B-NP) used in this study were prepared using an Endofree Plasmid Maxi Kit (QIAGEN, cat. No. 12362) according to the manufacturer's instructions. Six to seven-week-old BALB/c mice were immunized twice in both quadriceps by intramuscular (i.m) inoculation with 50 μg each of pGX27/B-NP or pGX27 vector at 2-week intervals. Electroporation (EP) was performed at a field strength of 50 V/cm as previously described to

improve vaccine delivery (17). The 'prime and deploy' groups were lightly anesthetized via isoflurane inhalation and administered 5×10^7 pfu rAd/mock or 5 µg CpG (TLR agonist ODN 1826; InVivoGen, San Diego, CA, USA) in 50 µl PBS by intranasal instillation 3 days after each immunization.

Virus and Infection

The B/Yamagata/16/88 (B/Yamagata, Yamagata lineage) virus was provided by Dr. Man Ki Song (International Vaccine Institute, Seoul, Korea). The B/Yamagata/16/88 virus was propagated in Madin-Darby canine kidney cells (ATCC CCL-34; ATCC, Manassas, VA, USA) as described previously (18,19). The precipitated virus was suspended in PBS and stored at −80°C. For challenge infection, mice were lightly anesthetized with isoflurane and intranasally administered 5 to 10 LD$_{50}$ of the virus.

ELISA

Sera were collected at indicated times to analyze Ab response. ELISA was used to determine the serum Ab titers against B-NP Ag. The B/Yamagata/16/88 was split with 0.5% Triton X-100 (Sigma, St. Louis, MO, USA), and 96F MaxiSorp Nunc-Immuno plates were coated with the split virus (3,600 pfu/well). The serum samples were diluted in 1% non-fat milk PBS and 0.05% Tween 20 and incubated for 2 h at room temperature (RT). After washing, we added HRP-conjugated rabbit anti-mouse IgG (Abcam) or HRP-conjugated goat-anti mouse IgA (Zymed Laboratories Inc., San Francisco, CA, USA) as a secondary Ab and incubated for 1 h at RT. The samples were then incubated with a 3,3′,5,5′-tetramethylbenzidine substrate and solution. The sample reaction was stopped with 1M phosphoric acid and detected at 450 nm by Thermo Multiskan EX (Vantaa, Finland).

Tissue harvest and intravascular staining

Spleens, lungs, and bronchoalveolar lavages (BAL) were collected after mice were sacrificed at indicated times with isoflurane/CO$_2$. Lungs were isolated from the trachea intact to perform BAL and gain BAL fluids. BAL cells were separated through centrifugation and resuspended in PBS with 0.5% FBS and 0.1% NaN3. Lung parenchymal cells were isolated after digestion with 1mg/ml collagenase type II (Worthington, Columbus, OH, USA) and 0.1% DNase I (Sigma) in RPMI 1640 supplemented with 10% FBS for 30 min at 37°C. They were then passed through a 70-µm cell strainer (SPL Life science, Pocheon, Korea). Spleens were homogenized using frosted glass, and red blood cells were lysed using Red Blood Cell Lysing Buffer (Sigma). Homogenous cell suspensions were prepared by passing tissues through a 70-um cell strainer (SPL Life science) and resuspended in IMDM or RPMI supplemented with 10% FBS. For intravascular immunostaining, monoclonal anti-CD45-PerCP/Cyanine 5.5 (clone 30-F11) (BioLegend, San Diego, CA, USA) was prepared at a 2 µg/200 µl concentration and injected intravenously through the tail vein. Animals were euthanized and tissues were isolated 5 min after injection.

Flow cytometry

Mononuclear cells were washed twice with cold PBS with 0.5% FBS and 0.1% NaN3 and centrifuged at 1,600 rpm for 5 min at 4°C. Purified rat anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA, USA) Fc blocker and 5 µg/ml streptavidin (Invitrogen) were added to each sample and incubated for 20 min at 4°C. The following cocktail was used for surface staining: CD3-APC/Cy7 (Clone 17A2; BioLegend), CD8-PE (Clone KT15; MBL Life science, Tokyo, Japan), CD62L-Alexa Fluor 700 (Clone MEL-14; BioLegend), CD69-PE/Cy7 (Clone H1.2F3; BioLegend), CD103-FITC (Clone 2E7; BioLegend) and influenza B-NP specific

D^d/NP$_{166-174}$ (FSPIRITFL) tetramer. The B-NP-specific D^d/NP$_{166-174}$ (FSPIRITFL) tetramer was produced as described previously (20). In brief, recombinant D^d protein was refolded in the presence of NP$_{166-174}$ peptide and β2m, biotinylated with biotin-protein ligase BirA, and then purified with Superdex-75 gel filtration column (GE Healthcare Life science, Chicago, IL, USA). The purified monomer was tetramerized with Alexa Fluor 647-Streptavidin conjugates (Thermo Fisher Scientific, Waltham, MA, USA). Stained cells were washed, fixed using BD FACS Lysing Solution (BD Pharmingen) at RT for 20 min, and collected on an LSRFortessa flow cytometer (BD Biosciences). Data were analyzed using Flowjo software (TreeStar Inc., Ashland, OR, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). A comparison between different study groups was statistically evaluated by an unpaired 2-tailed *t*-test, 1-way ANOVA, and 2-way ANOVA. Methods of comparison and corrections for multiple comparisons are indicated in the relevant figure legend. In all tests, statistical significance was quantified as $p < 0.05$, $p < 0.01$, and $p < 0.001$, and the figure represent mean±SEM.

RESULTS

Parenteral DNA vaccination alone fails to sufficiently protect against influenza B virus infection

To generate a DNA vaccine expressing a B-NP, the complete coding sequence of the NP gene was cloned into a pGX27 vector (**Fig. 1A**). The pGX27/B-NP plasmid was then transfected into HEK293 cells, and Western blotting was used to detect B-NP protein in the cell lysates and pellets of transfected cells (**Fig. 1B**, indicated by arrow). We then examined the immunogenicity and protective efficacy of pGX27/B-NP vaccination with EP in mice (**Fig. 1C**). The lethal challenge of the B/Yamagata virus indicated that the control and pGX27/B-NP DNA vaccine groups were poorly protected. All mice in both groups exhibited rapid weight loss and died within 5 days of the challenge (**Fig. 1D and E**). These results indicate that *in vivo* EP i.m immunization of the pGX27/B-NP failed to provide sufficient protection against pulmonary influenza B infection.

Recruitment of B-NP-specific CD8 T cells into the lung by intranasal delivery of non-replicating adenovirus vector

Vaccines expressing NP as a target Ag induce CTLs responses directed to conserved regions (21-25). i.m immunization of the DNA vaccine (pGX27/B-NP) did not sufficiently protect against influenza B challenge, despite the high IgG responses (**Supplementary Fig. 1**). Systemic immunization, such as i.m injection, generally primes circulating T$_{EM}$ and/or central memory T cells, rather than tissue-resident T$_{RM}$ cells. The tissue-CD8 T$_{RM}$ generally provide instant and robust protection against pathogens, especially in the lungs (3,10,26,27). Thus, it is likely that the failure of DNA vaccination to offer protection is due to its inability to induce CD8 T$_{RM}$ cells in the lungs.

Previous studies have shown that the prime-pull vaccine strategy could be used to generate T$_{RM}$ cells by recruiting effector and memory precursor cells to the target tissues during the priming phase (10,15,28,29). This vaccine strategy mostly employs prime-boost vaccination regimens with the same target Ags applied in different routes, in which boosting
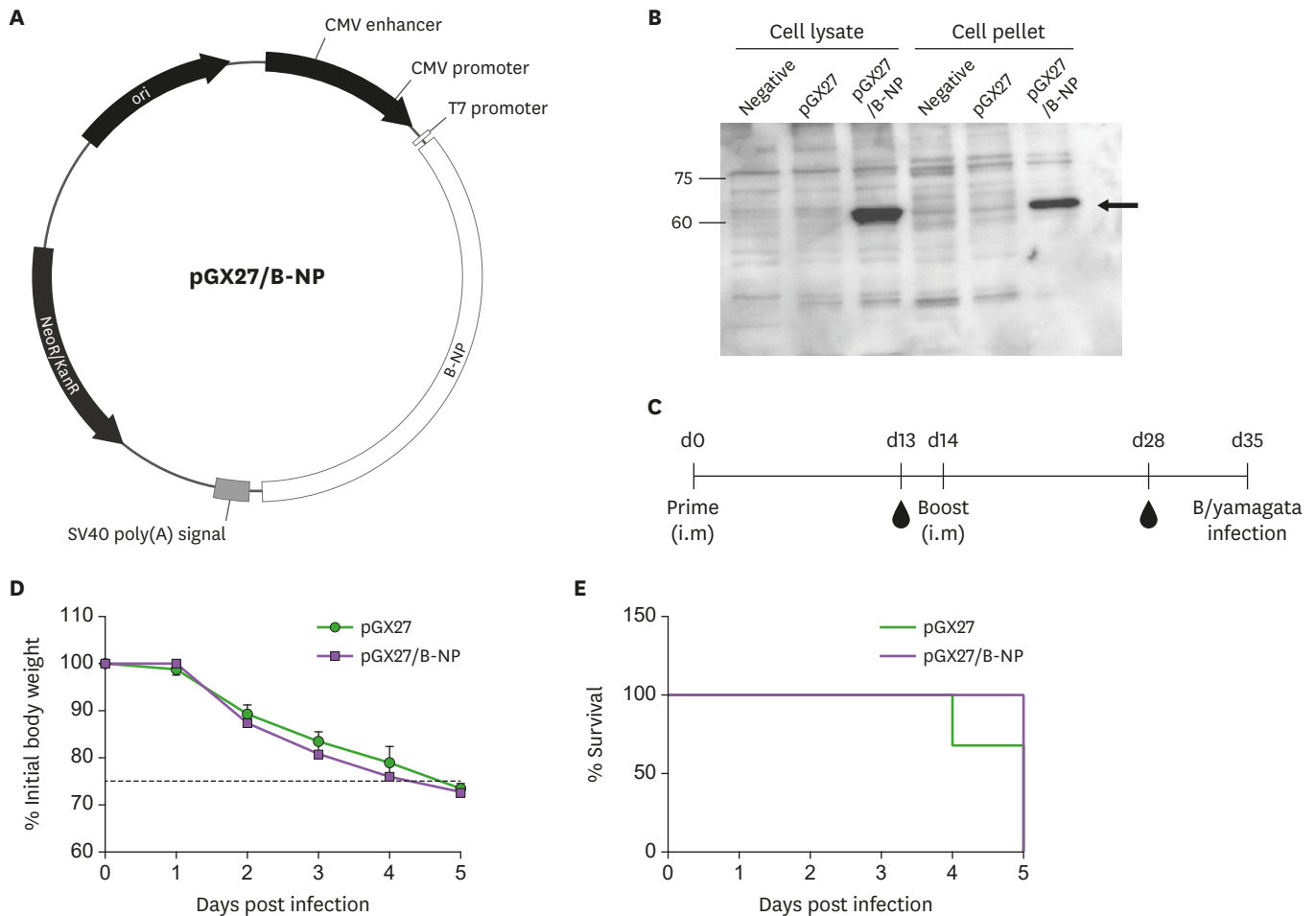
Figure 1. Construction of recombinant pGX27 expressing the B-NP and protection efficacy of DNA vaccine. (A) Schematic diagram of an B-NP in the pGX27 vector. The pGEM-T easy vector containing the NP gene of the B/Yamagata/16/88 virus was constructed and recombined with the pGX27 vector. (B) Validation of the DNA vaccine generated B-NP protein expression in the lysate (L) and supernatant (S) of cultured cells (arrow). HEK293 cells were transfected with pGX27/B-NP or rAd/B-NP as a positive control. We resolved the resulting cell lysate and supernatant by denaturing electrophoresis followed by western blotting. The cell lysates and supernatants yielded from the transfection of the corresponding empty vector were used as negative controls. (C) Experimental schema. BALB/c mice were immunized twice with 50 µg pGX27/B-NP or pGX27 via EP at 2 week intervals. Sera were collected at the indicated times. Five weeks after the prime, mice were challenged with 10 LD$_{50}$ B/Yamagata/16/88 by intranasal instillation. We then observed mortality and morbidity, weight loss (D), and survival rate (E) for 5 days after the 10 LD$_{50}$ challenge. Error bars represent mean±SEM.

immunization is directly applied to the target tissues. In our study, a different strategy was employed to assess whether Ag-independent inflammation with intranasally administered rAd/mock induces lung-resident CD8 T$_{RM}$ cells.

Mice were vaccinated twice at 2-week intervals with pGX27/B-NP, and rAd/mock or CpG was given intranasally 3 days after each DNA immunization to trigger inflammation in the respiratory tissues (**Fig. 2A**). CpG has been applied intranasally as a control reagent since it has been reported to mobilize CD8 T cells to the lungs in the systemic priming setting (1,9). All 3 immune groups exhibited similar B-NP-specific IgG responses (**Fig. 2B**). Mice were sacrificed after a boost immunization and lung tissues were harvested to analyze CD8 T-cell responses in the lung. To distinguish tissue-resident cells from circulating cells, intravascular labeling was performed with anti-CD45 monoclonal Ab 5 min before sacrifice for initial gating (**Supplementary Fig. 2**). The pGX27/B-NP + rAd/mock group exhibited the highest number of NP-specific CD8 T cells in the airway and the lung parenchyma, which was ≤20-
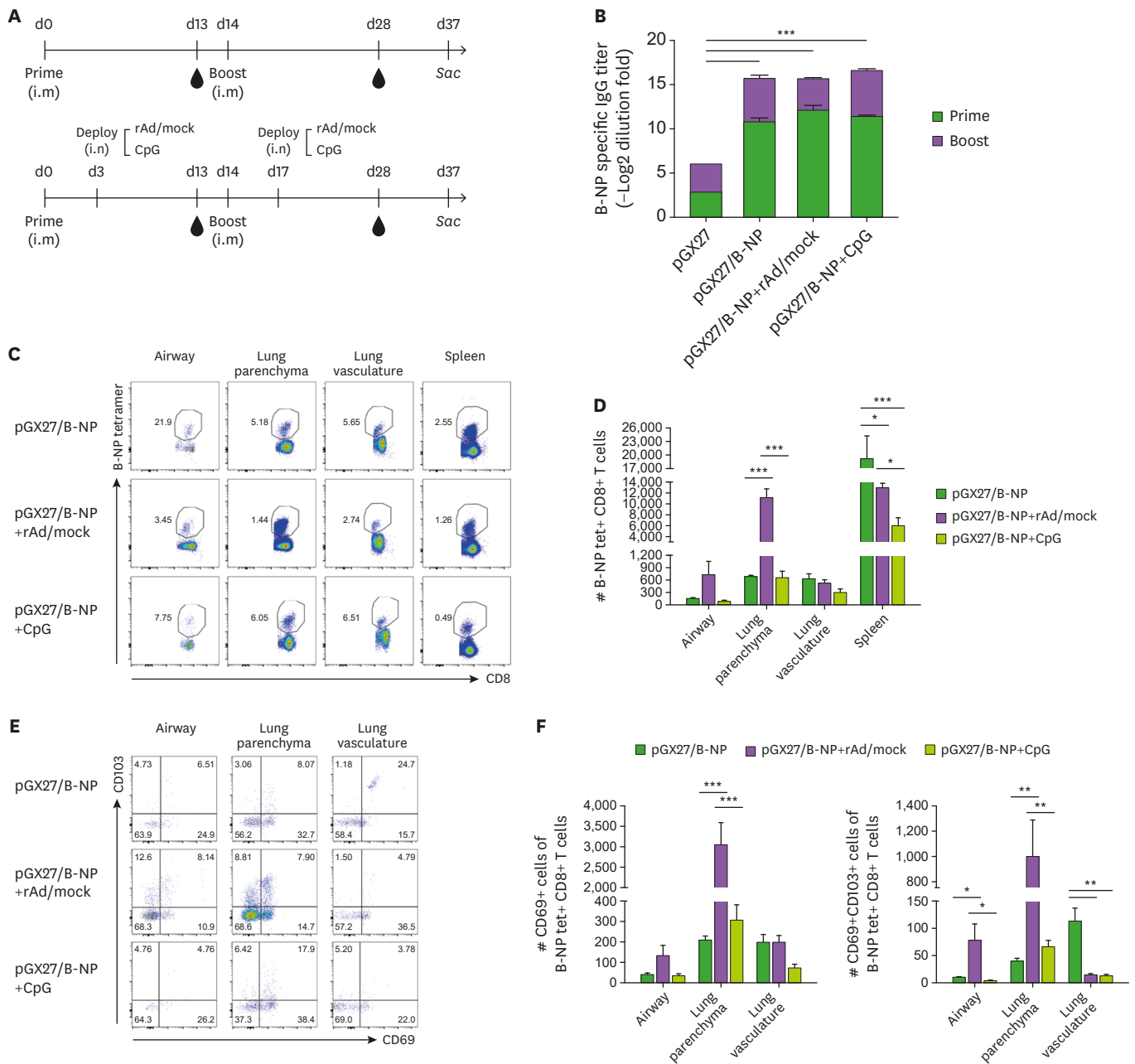
Figure 2. Induction of B-NP-specific lung CD8 T$_{RM}$ via the prime and deploy strategy during the effector phase of the T cell response. Female BALB/c mice were immunized twice with 50 μg of the pGX27/B-NP DNA vaccine via EP, and the 'prime and deploy' groups were administered with 5×10^7 pfu rAd/mock or 5 μg CpG intranasally 3 days after immunization. Sera were collected on days 13 and 28 to analyze the humoral immune response. We harvested spleens, lungs and BAL harvested from individual animals 37 days after the first immunization. BALB/c mice were injected with 2 μg of fluorescently labeled anti-CD45 monoclonal Ab 5 min before euthanasia and tissue harvest. (A) Timeline for vaccine administration. Mice were immunized twice with pGX27/B-NP only (top) and received rAd/mock intranasally or CpG 3 days after immunization (bottom). (B) B-NP-specific IgG titers were determined by ELISA. The data represent the mean (Log$_2$ endpoint titer)±SEM values of 4 mice per group. Statistical comparisons were performed using a 2-way ANOVA with Bonferroni post-tests. (C) Representative flow plots of B-NP specific CD8 T cells from the airway, lung parenchyma, lung vasculature, and spleen. The pGX27/B-NP DNA vaccine group (top), pGX27/B-NP + rAd/mock (middle), and pGX27/B-NP + CpG (bottom). (D) The total number of B-NP specific CD8 T cells in the airway, lung parenchyma, lung vasculature, and spleen. Error bars represent mean±SEM. Statistical comparisons were performed using a 2-way ANOVA with Bonferroni post-tests. (E) Representative flow plots of CD69 and CD103 expression in B-NP-specific CD8 T cells in the airway, lung parenchyma, and lung vasculature. (F) Bar graphs showing the total number of B-NP-specific CD8 T cells expressing CD69+ (left) and CD69+CD103+ (right) in the airway, lung parenchyma, and lung vasculature. Error bars represent mean±SEM. Statistical comparisons were performed using a 1-way ANOVA with Newman-Keuls Multiple comparison test.
*p<0.05, **p<0.01, ***p<0.001.

fold higher in the lung parenchyma than it was in the pGX27/B-NP group (**Fig. 2C and D**). On the other hand, the pGX27/B-NP + CpG mice had similar Ag-specific CD8 T cells as the pGX27/B-NP group. Likewise, the pGX27/B-NP + rAd/mock group exhibited significantly increased numbers of NP-specific CD69+ and CD69+CD103+ T$_{RM}$ cells compared to the pGX27/B-NP group (**Fig. 2E and F**). These results demonstrated that intranasal delivery of a recombinant adenovirus expressing no Ag recruits Ag-specific CD8 T cells to the lungs. This suggests lung-resident CD8 T cells are established by the 'prime-deploy' strategy in the absence of cognate Ag.

Ag-independent 'prime-deploy' strategy enhances the protective efficacy of the DNA vaccine against Influenza B infection

To determine whether our 'prime-deploy' strategy for recruiting lung T$_{RM}$ cells could provide enhanced protection, immunization was performed on the same schedule as in **Fig. 2**, and mice were challenged with influenza B virus (B/Yamagata/16/88) (**Fig. 3A**). The pGX27/B-NP + rAd/mock group showed minimum weight loss, whereas the pGX27/B-NP group showed more rapid weight loss until 5 days after infection (**Fig. 3B**). The pGX27/B-NP + rAd/mock group had complete protection, while the group with the DNA vaccine alone (pGX27/B-NP) had a 25% survival rate (**Fig. 3C**). Interestingly, lung virus titers were still detected in all groups at day 2 and 4 post-challenge though the titers were lower in pGX27/B-NP + rAd/mock group than pGX27/B-NP group (data not shown). It is likely that NP-specific CD8 T cells mediate the protection which may not necessarily correlate with virus titers detected in the lungs upon lethal challenge (25).

To analyze Ag-specific CD8 T cells after challenge, the survivors in each group were sacrificed on day 14. The pGX27/B-NP and pGX27/B-NP + CpG group had higher numbers of NP-specific CD8 T cells than the pGX27/B-NP + rAd/mock group in all examined tissues (**Fig. 3D and E**). Further, when NP-specific CD8 T cells were gated and analyzed for T$_{RM}$ markers such as CD69 and CD103, the number of CD69+ and CD69+CD103+ CD8 T cells were higher in the pGX27/B-NP and pGX27/B-NP + CpG groups (**Fig. 3F and G**). These results suggest that it is not the magnitude of the secondary CD8 T-cell response, but rather the existing lung-resident CD8 T$_{RM}$ cells that provide protection.

Kinetics of airway and lung parenchymal CD8 T$_{RM}$ cells

To examine the kinetics of CD8 T cells, we analyzed the absolute number of NP-specific CD8 T cells in the airway and lung parenchyma at days 37 and 64. In the airway, the population of NP-specific CD8 T cells drastically decreased in the group with the DNA vaccine alone (pGX27/B-NP) from day 37 to day 64, whereas the cells in the pGX27/B-NP + rAd/mock group increased (**Fig. 4A**). The population of CD69+CD103+ T$_{RM}$ showed similar kinetics. However, CD8 T cells in the lung parenchyma had different kinetics from those in the airway. NP-specific CD8 T$_{RM}$ cells in the lung parenchyma gradually declined over time, regardless of the vaccination strategy (**Fig. 4B**), consistent with previous research in which lung parenchymal CD8 T$_{RM}$ cells had a limited half-life compared to other tissues due to apoptosis and possible cell migration to the airway (30,31). An increase of CD8 T cells in the airway might result from their migration from the lung parenchyma, as previously reported (32-34).
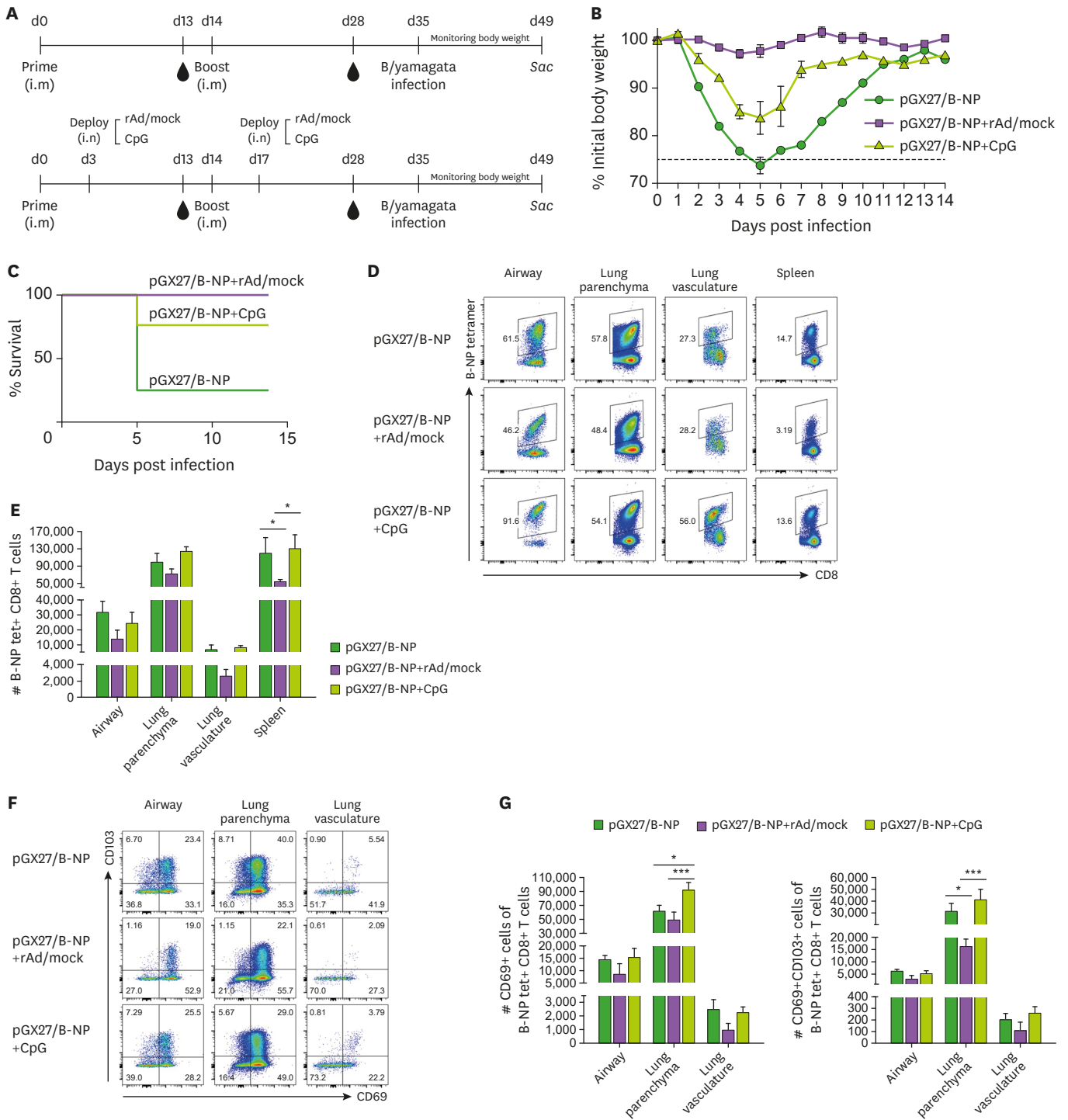
Figure 3. Protection efficacy of 'prime and deploy' vaccination strategy against influenza B. (A) Schematic representation. Three weeks after the last immunization, mice were challenged with 5 LD$_{50}$ B/Yamagata/16/88. We scored mortality and morbidity for 2 weeks and sacrificed (isoflurane/CO$_2$) the animals. We tracked weight loss (B) and the survival rate (C) over 14 days after the 5 LD$_{50}$ challenge. Error bars represent mean±SEM. (D) Representative flow plots show the B-NP tetramer+ CD8 T cells frequency in the airway, lung parenchyma, lung vasculature, and spleen. (E) The total number of B-NP-specific CD8 T cells in the airway, lung parenchyma, lung vasculature, and spleen. Error bars represent mean±SEM. Statistical comparisons were performed using a 2-way ANOVA with Bonferroni post-tests. (F) Representative flow plots of B-NP-specific CD8 T$_{RM}$ after influenza B infection. (G) Bar graphs show the total number of B-NP-specific CD8 T cells expressing CD69+ (left) and CD69+CD103+ (right) in the airway, lung parenchyma, and lung vasculature. Error bars represent mean±SEM. Statistical comparisons were performed using a 2-way ANOVA with Bonferroni post-tests.
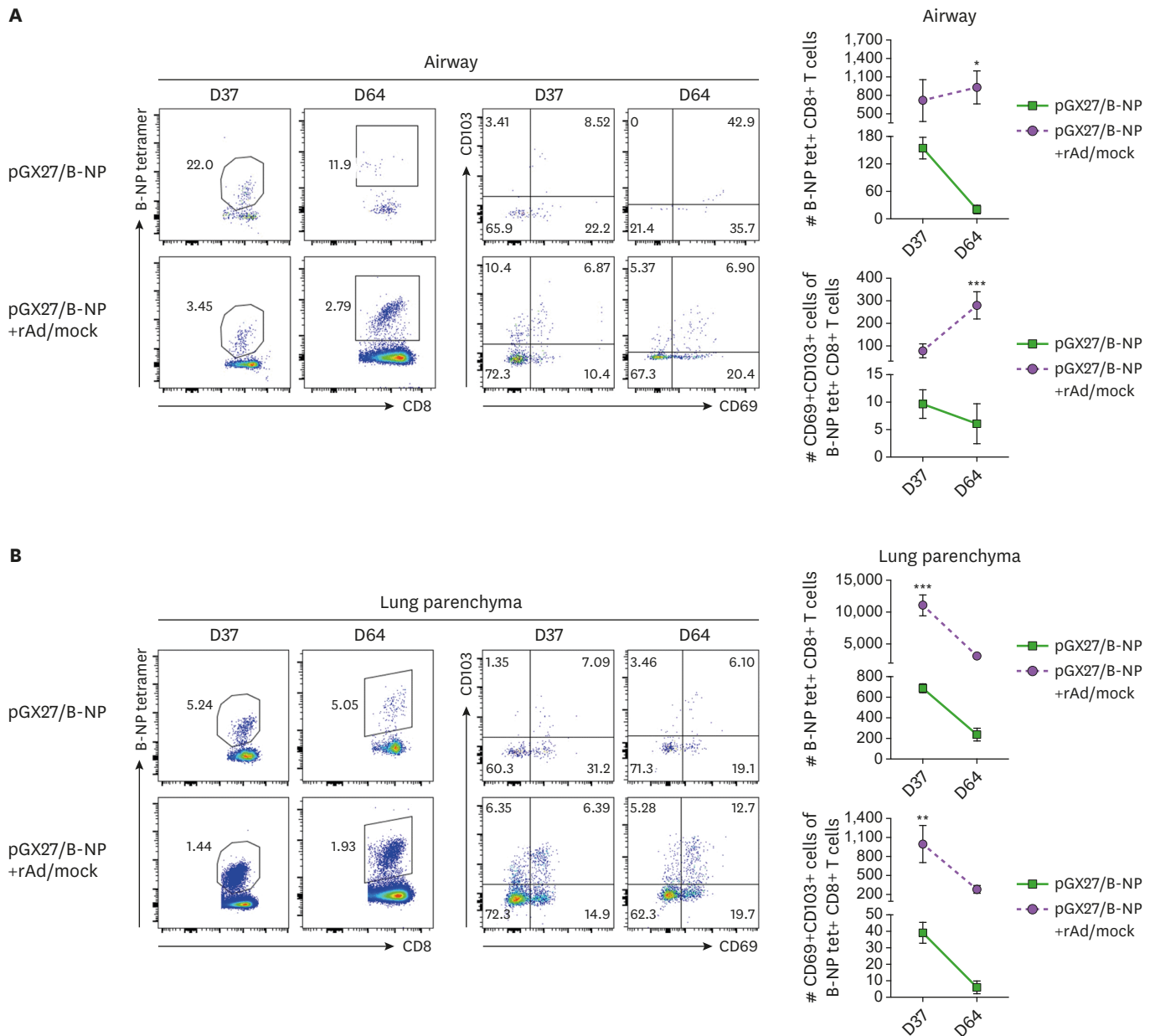*p<0.05, ***p<0.001.

Figure 4. CD8 T$_{RM}$ in the lung parenchyma, but not in the airway, established by a 'prime and deploy' vaccination strategy decreases over time. BALB/c mice were sacrificed at specified time points (day 64) and NP-specific CD8 T-cell kinetics were compared to the group of mice who were sacrificed on day 37. Mononuclear cells from BALs and lungs were stained with B-NP tetramer and a CD8 T$_{RM}$ surface marker. (A) Schematic representation. (B) Representative flow plots of B-NP-specific CD8 T$_{RM}$ in the airway. pGX27/B-NP (top), pGX27/B-NP + rAd/mock (bottom). Line graphs represent kinetic changes of the B-NP-specific CD8 and CD69+CD103+ T cells in the airway. Error bars represent mean±SEM. Statistical comparisons were performed using a 2-way ANOVA with Bonferroni post-tests. (C) Representative flow plots of B-NP-specific CD8 T$_{RM}$ in the lung parenchymal tissue. pGX27/B-NP (top), pGX27/B-NP + rAd/mock (bottom). Line graphs represent kinetic changes of the B-NP-specific CD8 and CD69+CD103+ T cells in the lung parenchyma. Error bars represent the mean± SEM. Statistical comparisons were performed using a 2-way ANOVA with Bonferroni post-tests.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

DISCUSSION

We investigated the immunogenicity and protective efficacy of intramuscularly-administered DNA vaccine expressing theB-NP. We found that DNA vaccination elicits humoral and systemic CD8 T-cell responses, but fails to confer sufficient protection against an influenza

B challenge. Nonetheless, intranasal delivery of a non-replicating adenovirus vector that expresses no Ag after DNA vaccination provided enhanced protection, which is mediated by the active recruitment of NP-specific CD8 T$_{RM}$ cells in the lungs.

NP-expressing DNA vaccination alone is not sufficient to protect against respiratory challenge infection, even it induced serum IgG and systemic memory CD8 T cells. This suggests that parenteral NP DNA vaccination could not induce protective mucosal immunity, which recognizes and eliminates infected cells in the early phase. Memory T cells deposited in the respiratory tract (T$_{RM}$) play a critical role in this local protection by direct killing and releasing cytokines (35). After encounters with the influenza virus, lung T$_{RM}$ cells might rapidly respond, mediating enhanced viral clearance and survival.

The second-generation non-replicating adenovirus 5 vector used in our study (rAd/mock) exhibited strong 'deployment' effects to induce lung-resident CD8 T$_{RM}$ responses. The adenoviruses stimulate innate immunity via pathogen-associated molecular patterns, such as viral capsid proteins, cytosolic DNA, and subsequently, IFNs and proinflammatory cytokines such as IL-6, IL-12, and TNF-α, which promote innate immunity (36,37). The mucosal delivery of recombinant adenovirus vector also induced long-lasting innate immunity in the lungs (38). In addition, the interaction of Ad5 fiber protein with the coxsackie-adenovirus receptor activates signaling pathways *in vitro*, resulting in the IL-6 expression (39). Various membrane-associated kinase signals are also triggered during Ad binding and entry into host cells (40), some of which help induce the early innate immune responses (41-45). Thus, mucosal administration of rAd/mock might have induced various pro-inflammatory and inflammatory events in the respiratory system, deploying primed CD8 T cells into the lungs. Further studies should investigate the detailed mechanisms of this adenovirus-induced immune remodeling.

Our 'prime and deploy' vaccination strategy is different from the previously described 'prime and pull' strategy because there is no cognate Ag in the 'pulling' process. NP-expressing DNA vaccine may systemically prime NP-specific CD8 T-cell responses, and subsequent triggering of local inflammation in the respiratory tract might 'deploy' CD8 T cells non-specifically to the lungs. This 'prime and deploy' strategy increased NP-specific CD8 T$_{RM}$ cells and the overall number of CD8 T cells in the airways and lung parenchyma as compared to DNA vaccination alone. The majority of NP-specific CD8 T cells established by the 'prime and deploy' strategy express markers typical of CD8 T$_{RM}$ cells, such as CD69 and CD103, suggesting that some of these CD8 T cells eventually develop into tissue-resident cells. These CD8 T$_{RM}$ cells may play a crucial role in protecting against subsequent challenges, as they might offer frontline surveillance and rapid control over respiratory infections (26,27,46,47).

Our results suggest that the 'deploy' strategy with non-replicating adenovirus vectors could be applied to systemic vaccination regimens, as it is Ag-independent. Furthermore, non-replicating adenovirus-based mucosal vaccines could deploy existing systemic memory T cells to the tissues where the adenovirus vector is administered. Selecting suitable immunization routes may be key to the general 'deploy' effects of adenovirus vector-based vaccines.

In conclusion, we have shown that the DNA vaccine 'priming' and Ag-independent 'deploy' strategy via mucosal adenovirus vector delivery established CD8 T$_{RM}$ in the pulmonary tissues and enhanced protection against influenza B infection. Current influenza vaccines are mostly i.m, so it is difficult to generate T$_{RM}$ and mucosal immunity. The 'prime and deploy' strategy in this study could be further developed as a universal vaccine that covers a broader range of virus strains.

ACKNOWLEDGEMENTS

SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Comparison of B-NP specific humoral immune response following DNA vaccination. Female BALB/c mice were immunized twice with 50ug pGX27/B-NP or empty vector (pGX27) via EP. Sera were collected at the indicated days. Influenza B-NP-specific IgG titers were determined by ELISA. The data represent the mean (Log$_2$ endpoint titer)±SEM values of 4 mice per group. Statistical comparisons were performed using a 1-way ANOVA with Bonferroni's multiple comparison test.

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Supplementary Figure 2

Gating strategy to identify Ag-specific CD8 T$_{RM}$ cells in the lung parenchyma and lung vasculature. Experimental design was same as **Figure 2A**. BALB/c mice were vaccinated as indicated schedule. Mice were injected with 2 µg fluorescently labeled anti-CD45 monoclonal Ab 5 min before euthanasia and tissue harvest. Bottom left panel showing CD45$^{+/-}$ cells gated out of CD3+CD8+ population. The lung parenchyma and vasculature were distinguished by i.v staining through tail vein. Bottom right panels showing surface expression of B-NP specific CD69 and CD103 markers on CD45 positive and CD45 negative cells in the lung parenchyma and lung vasculature of these mice.

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