

Brief Communication



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Conflict of Interest

The authors declare no potential conflicts of
interest.

Abbreviations

BE, B-cell epitope; EID50, 50% egg infectious
dose; HA, hemagglutinin; IFAV, influenza A
virus; IFV, influenza virus; iM2Pr, inverted
nM2Pr; M2, matrix protein 2; M2e, ectodomain
of M2; MLD50, 50% mouse lethal dose; NA,
neuraminidase; nM2e, N-terminal sequence
of M2e; nM2HK, nM2e sequence for A/Hong

Evaluation of Protective Immunity of Peptide Vaccines Composed of a 15-mer N-terminal Matrix Protein 2 and a Helper T-Cell Epitope Derived from Influenza A Virus

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ABSTRACT

The matrix protein 2 of influenza A virus (IFAV) has a relatively conserved ectodomain (M2e) composed of 23 amino acids, and M2e-based vaccines have been suggested to induce broad protective immunity in mice. In this study, we investigated whether N-terminal sequence of M2e (nM2e)-based vaccines with more conserved nM2e could induce influenza viral neutralizing activity. We constructed linear peptide vaccines with an nM2e sequence for PR8 virus (nM2Pr) connected to a probable 17-mer IFAV-derived helper T-cell epitope (ThE: T1, T2, or T3) at its N- or C-terminus. The peptide vaccines induced significant production of nM2e Abs regardless of either type or location of the ThE-epitope in BALB/c mice, while only T3 was effective in C57BL/6 mice. The Abs against nM2Pr-T3 elicited broader binding affinities to the nM2e peptides derived from various IFAVs than those against T3-nM2Pr. In addition, the nM2e-based vaccines efficiently protected the immunized mice from the lethal challenge of PR8 virus. These results suggest that the more conserved nM2e without cysteine will be useful for development of universal peptide vaccines than M2e.

Keywords: Influenza Vaccines; M2 protein, Influenza A virus; Vaccines, Subunit; ELISA; Antibodies, Neutralizing

INTRODUCTION

Influenza A viruses (IFAVs) are important respiratory pathogens causing high morbidity and mortality in human and avian (1). In accordance to a World Health Organization report, IFAVs typically infect 10 to 20% of the total worldwide population, and results in 2 to 5 million severe cases and 250,000 to 500,000 deaths per year. In addition, new pandemic influenza viruses (IFVs) are emerging irregularly when novel IFAV subtypes introduced into the human population obtained the efficient human-to-human transmission ability. Pandemic IFAVs usually showed more severe symptoms than seasonal IFAVs (2-5).

Kong/156/97 (H5N1) virus; nM2K1, nM2e sequence for A/Korea/01/2009 (H1N1) virus; nM2K2, nM2e sequence for A/chicken/Korea/IS/2006 (H5N1) virus; nM2K3, nM2e sequence for A/wild bird/Korea/A81/2009 (H5N2) virus; nM2Pr, nM2e sequence for PR8 virus; NP, nucleoprotein; PB1, polymerase basic 1; STBS, skim milk in tris-buffered saline; TBS, tris-buffered saline; ThE, helper T-cell epitope

Author Contributions

Conceptualization: Lee MK; Formal analysis: Kim JK, Lee MK; Investigation: Kim JK, Cheong S, Lee MK; Supervision: Lee MK; Writing - original draft: Kim JK, Cheong S, Lee MK; Writing - review & editing: Kim JK, Lee MK.

IFVs are RNA viruses with a segmented genome. IFAVs have been known to cause pandemics, and are infectious into various animals including humans, pigs, birds, and so on (6). Two major glycoproteins, hemagglutinin (HA; 18 subtypes) and neuraminidase (NA; 11 subtypes), determine the host specificity and virulence (7). The HA proteins inducing IFAV infection are primary targets for IFV vaccine development, but constantly change because of the high error rate of the viral RNA replicase (8). Therefore, seasonal IFAV vaccine strains should be routinely updated when IFAVs acquire mutations under the immune pressure of circulating Abs called antigenic drift (5,9). Due to the problem, the need for development of universal vaccines is constantly emerging (5).

The IFAV matrix protein 2 (M2) is a proton channel protein composed of 97 amino acids (10,11), which is a target of anti-IFAV drug (amantadine derivatives) (12). It is present at low copy numbers on the virion surface, while is highly expressed on the plasma membrane of infected cells (10). Furthermore, it has very low immunogenicity in the IFAV infected organism because of the small size and probable shielding by much larger glycoproteins, HA and NA. The ectodomain of M2 (M2e) is composed of 23 amino acids and relatively conserved. The anti-M2e Abs generated by immunization of the M2e fused to the hepatitis B core protein and helper T-cell epitope (ThE) peptide showed broad neutralizing activities against IFAVs (13,14). The protective immunity of anti-M2e Abs is known to be induced by the Ab dependent-cell-mediated cytotoxicity, complement dependent cytotoxicity, and/or prevention of virus release (10,15,16).

In this study, we select a short N-terminal sequence of M2e (nM2e) composed of 15 amino acids as a B-cell epitope (BE), which is more conserved and no cysteine residue as compared with the whole M2e sequence. We also selected 3 17-mer ThEs derived from conserved M1, nucleoprotein (NP), and polymerase basic 1 (PB1) proteins of IFAV (17), chemically synthesized the linear peptide immunogens with the BE-ThE or ThE-BE arrangement, and immunized into BALB/c or C57BL/6 mice. We investigated production and specificity of the anti-nM2e Abs in BALB/c or C57BL/6 mice and survivals of the immunized mice from lethal virus challenge in C57BL/6 mice.

MATERIALS AND METHODS

Animals

The BALB/c (BALB/cAnNHsd) and C57BL/6 (C57BL/6N) mice were supplied from the animal facility and maintained in the specific pathogen free facility under guidelines on the operation of animals of Korea Research Institute of Bioscience and Biotechnology (KRIBB). All the experiments using mice were approved by KRIBB-Institutional Animal Care and Use Committee (IACUC; approval No. KRIBB-AEC-11105) and performed in accordance with the IACUC guidelines to ensure the welfare of the experimental animals.

Peptide synthesis

The peptide immunogens and Ags were synthesized by the Fmoc-based solid-phase peptide synthesis using the standard dicyclohexylcarbodiimide/1-hydroxybenzotriazole method. The peptides were purified by using a C₁₈ reverse-phase HPLC (Waters, Milford, MA, USA) and analyzed using MALDI-TOF (Shimadzu Axima Plus, Kyoto, Japan). The synthesized peptides are summarized in **Table 1**.

Table 1. The 1 letter amino acid sequences of peptides used as immunogens and Ags

Peptides	Sequences	Remarks
Immunogens		
nM2Pr-T1	<u>MSLLTEVETPIRNEW</u> [*] <i>GTNPLIRHENRMVASTT</i> [†]	T1: M1 (169–185)
T1-nM2Pr	TNPLIRHENRMVASTT <i>G</i> [†] <u>MSLLTEVETPIRNEW</u> [*]	(16) [§]
nM2Pr-T2	<u>MSLLTEVETPIRNEW</u> [*] <i>GVLYDKEEIRRIWRQANN</i> [†]	T2: NP (109–125)
T2-nM2Pr	<i>VLYDKEEIRRIWRQANN</i> [†] <u>GMSLLTEVETPIRNEW</u> [*]	(16) [§]
nM2Pr-T3	<u>MSLLTEVETPIRNEW</u> [*] <i>GLQLFIKDYRYTYRCHRG</i> [†]	T3:PB1 (548–564)
T3-nM2Pr	<i>LQLFIKDYRYTYRCHRG</i> [†] <u>GMSLLTEVETPIRNEW</u> [*]	(16) [§]
Ags		
nM2Pr	<u>MSLLTEVETPIRNEW</u> [*] GGC [‡]	(17) [§]
iM2Pr	CGG [‡] <u>MSLLTEVETPIRNEW</u> [*]	Inverted nM2Pr
nM2K1	<u>MSLLTEVETPTRSEW</u> [*] GGC [‡]	(19) [§]
nM2K2	<u>MSLLTEVETPTRNEW</u> [*] GGC [‡]	(20) [§]
nM2K3	<u>MSLLTEVETPTRNGW</u> [*] GGC [‡]	(21) [§]
nM2HK	<u>MSLLTEVELTRNGW</u> [*] GGC [‡]	(22) [§]

^{*}The underlined sequences represent the BE derived from the M2 proteins; [†]The italicized sequences represent the ThEs derived from the indicated influenza A viral proteins in remarks; [‡]The additional GGC and CGG sequences of Ag peptides were introduced to provide a flexible space (GG) and a sulfhydryl group (C) for BSA conjugation; [§]The amino acid sequences of the B- and ThEs were obtained from the indicated references in remarks.

Peptide conjugation to BSA

The nM2e peptide Ags were conjugated to a carrier protein, BSA, using N-(γ -maleimidobutyryloxy) succinimide ester for sulfhydryl-amine conjugation according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). The peptide-BSA conjugates were used as coating Ags for ELISA to analyze anti-nM2e Ab production and reactivity.

Ab production and ELISA

50 μ g of nM2e sequence for PR8 virus (nM2Pr)-ThE or ThE-nM2Pr (**Table 1**), was emulsified with the Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and immunized 4 times at 2 wk intervals into 6 to 7 wk old BALB/c or C57BL6 mice by intraperitoneal injection. After 2 wk of final injection, antisera were obtained by centrifugation of bloods bleeding from retro-orbital sinus. Production and specificity of anti-nM2Pr Abs in antisera were analyzed by indirect ELISA against the nM2e-BSA conjugates. Briefly, 96 well microtiter plates were coated with 50 ng peptide-BSA streptavidin in 50 μ l coating buffer (0.1M sodium carbonate, pH 9.6) for 1 h and blocked with 1% skim milk in tris-buffered saline (TBS) (STBS; 20mM Tris and 150 mM sodium chloride, pH 7.5). After washing the plates with the washing solution (0.1% Tween 20 in TBS), the plates were incubated with the serially diluted antiserum in STBS. The bound Abs in the immunized mice were measured using peroxidase conjugated anti-mouse IgG rabbit Ab and 3,3',5,5'-tetramethylbenzidine-substrate system (BD Biosciences, Franklin Lakes, NJ, USA). After stopping the reaction by adding 1/2 volume of 2.5 M sulfuric acid, the color development was monitored at 450 nm.

Virus preparation and neutralizing assay

The PR8 virus was used as a challenge virus in this study. The virus was grown in 10-day-old embryonated chicken eggs for 48 h at 35°C. The allantoic fluid was then harvested and stored at -80 °C until use. The virus titers, the 50% egg infectious dose (EID₅₀) and the 50% mouse lethal dose (MLD₅₀) of the virus stock, were determined by infecting ten-fold serially-diluted viruses into the embryonated chicken eggs and the C57BL/6 mice, respectively. Groups of C57BL/6 mice were immunized with either nM2Pr-T3, T3-nM2Pr, or PBS (negative control) as described above. Two wk after the final immunization, all the immunized mice were challenged intranasally with 30 μ l of 10^{5.62} EID₅₀/ml (10 MLD₅₀) of PR8 virus. The weights of

all mice were monitored daily for 14 days, and the mice with a weight loss of more than 25% were considered to be dead.

RESULTS AND DISCUSSION

Design and synthesis of immunogen and Ag peptides

To develop an efficient IFAV vaccine, the peptide vaccine should have both BE and ThE in a molecule. We hypothesized that the 15-mer nM2e BE might have more beneficial effects than the whole 23-mer M2e BE with 2 cysteine residues if it provides a protective immunity, since the nM2e BE is shorter and more conserved than the M2e BE as well as has no cysteine residue. The M2e BE has 2 cysteine residues, which have a possibility to form disulfide bond(s) between the M2e molecules. If the protective immunity of an M2e vaccine is affected by the disulfide bond(s), it may be difficult to control the M2e vaccine quality during storage. Therefore, the nM2e-based vaccine is considered more effective if it provides a protective immunity. To evaluate the *in vivo* nM2e-based vaccine efficacy, we designed the linear peptide vaccines with either BE-ThE or ThE-BE arrangement (**Table 1**). We selected nM2Pr derived from the PR8 virus (A/Puerto Rico/8/1934, H1N1) as a BE (18). We also selected 3 conserved ThEs (T1, T2, and T3) derived from conserved M1, NP, and PB1 proteins of IFAV, respectively, that were reported to induce strong reactivity to at least one or more human class II MHC proteins (HLA-DRs) but not to class I ones (17).

To examine the IFAV stain-specific reactivity of the anti-nM2Pr Abs, we synthesized the nM2e Ag peptides (**Table 1**), nM2K1, nM2K2, nM2K3, and nM2HK derived from A/Korea/01/2009 (H1N1) (19), A/chicken/Korea/IS/2006 (H5N1) (20), A/wild bird/Korea/A81/2009 (H5N2) (21) and A/Hong Kong/156/97 (H5N1) (22), respectively, in addition to the PR8 virus derived peptides, nM2Pr and iM2Pr (**Table 1**). The Ag peptides were conjugated to BSA to enhance the coating efficiency on the ELISA plate.

Ab production of nM2e peptide vaccines in mice

Fig. 1 shows the ELISA profiles of anti-nM2Pr Abs produced in BALB/c and C57BL/6 mice against nM2Pr-BSA and iM2Pr-BSA. Interestingly, the anti-nM2Pr Abs significantly produced in the immunized BALB/c mice regardless of the ThE types and arrangements. Moreover, the Abs showed the similar reactivity to both nM2Pr and iM2Pr peptides, indicating that the Abs produced in BALB/c mice predominantly recognize the internal nM2Pr sequence rather than the terminal one. However, the peptide vaccines showed the different efficiency in C57BL/6 mice depending on the ThEs. T1 and T2 were not effective in C57BL/6 mice, especially those with the nM2Pr-ThE arrangement. In contrast, T3 was relatively effective, especially that with the T3-nM2Pr arrangement. The BALB/c mouse has 2 class II MHC, I-A^d and I-E^d, while the C57BL/6 mouse has only I-A^b. T3 was reported to enable broad interaction with the human class II MHCs, DR2, DR3, and DR4, on ELISPOT assay, while T1 and T2 limitedly interact with only DR3 (17). We assume that the DR3-limited T1 and T2 may have little affinity to the I-A^b of C57BL/6, but strong affinity to the I-A^d and/or I-E^d of BALB/c. In contrast, T3 that interacts with all the human class II MHCs is inferred to have also broader affinities to the mouse ones. The similar result was reported in a previous study (23). The results suggest that development of broad and powerful ThEs are essential for developing effective M2e-based peptide vaccines.

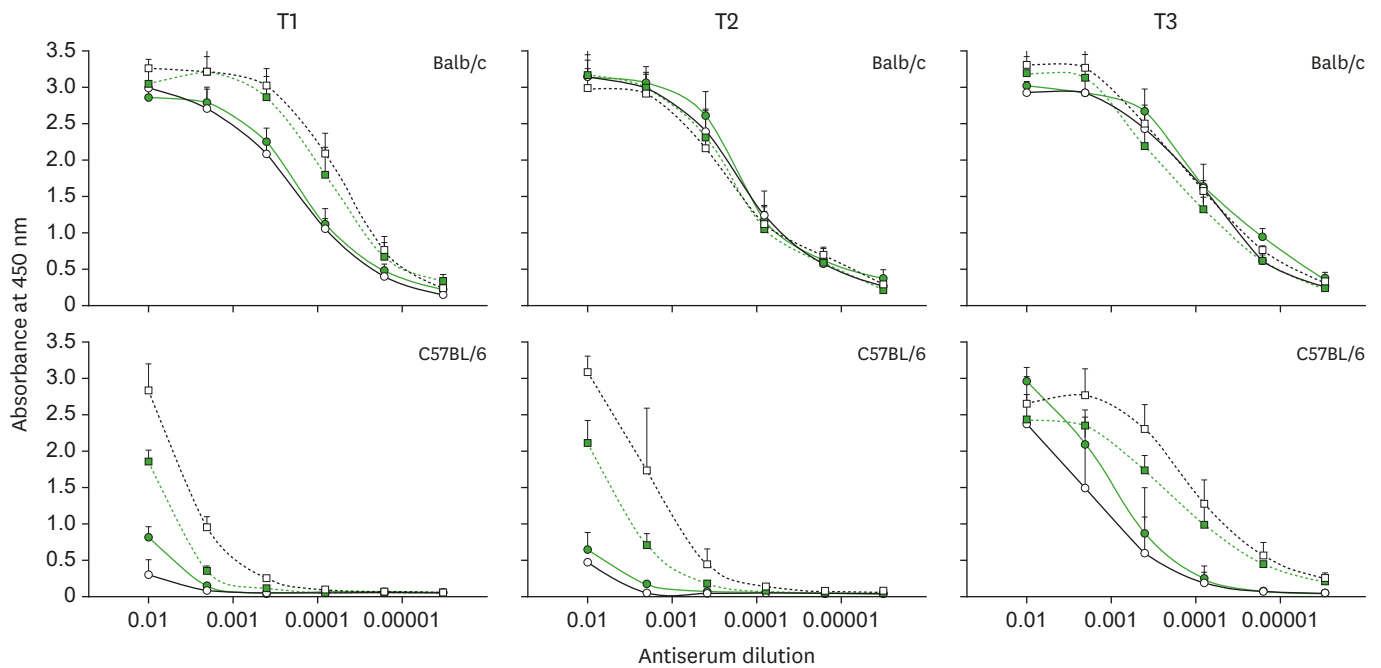


Figure 1. ELISA profiles of anti-nM2Pr Abs produced in BALB/c and C57BL/6 mice. The Abs were produced by immunization of nM2Pr-The (circle) and The-nM2Pr (square) as described in “materials and methods”. ELISA assays were performed against the coating Ags, nM2Pr-BSA (closed) and iM2Pr-BSA (open). T1, T2 and T3 represent the Th-cell epitopes (ThEs) used in this study. The sequences of peptide immunogens and Ags are represented in **Table 1**.

Virus specific antigenicity of anti-nM2Pr Abs

We further investigated the IFAV strain-specificity of Abs produced by immunization of nM2Pr-T3 or T3-nM2Pr in BALB/c or C57BL/6 mice (**Fig 2A**). The Ab titers shown in **Fig. 2B** were determined by the equation ($=1/\text{dilution of antisera at absorbance } 0.5$). The Abs generated by immunization of nM2Pr-T3 in BALB/c mice revealed similar affinities to all the nM2e peptide-BSA conjugates as well as iM2Pr-BSA, indicating that the Abs predominantly recognize the common internal sequence including MSLLTVEVT at the N-terminus of the nM2e peptides. The nM2Pr-T3 immunization in C57BL/6 mice induced 80% less Ab production than that in BALB/c mice, and the resulting Abs showed very similar affinity to the nM2e peptides except nM2HK with Pro10Leu substitution. In contrast, the Abs generated by immunization of T3-nM2Pr with a free C-terminus of nM2Pr exhibited the reduced affinities to the peptide Ags with substituted residues (the bold characters in **Table 1**), regardless of immunized mice (**Fig. 2**). Taken together, the results demonstrate that the anti-nM2Pr Abs generated by T3-nM2Pr immunization predominantly recognize the variable C-terminus of nM2Pr. In this respect, the peptide immunogen with the nM2e-The arrangement is considered more useful for development of universal peptide vaccine, even though it shows less immunogenicity in C57BL/6 mice. We expect that the problem can be overcome by development of broad and potent The peptides and effective adjuvants.

In vivo protective immunity of peptide immunogens against lethal virus challenge

Protective immunity of the peptide immunogens is essential for evaluation of vaccine efficacy. We challenged PR8 virus into the C57BL/6 mice with or without immunization of either nM2Pr-T3 or T3-nM2Pr and monitored for weight loss for 14 days after virus challenge. **Fig. 3A** shows the peptide immunogen-specific responses of the individual antisera to either nM2Pr-BSA or iM2Pr-BSA. **Fig. 3B and C** show the body weight changes and survival rates,

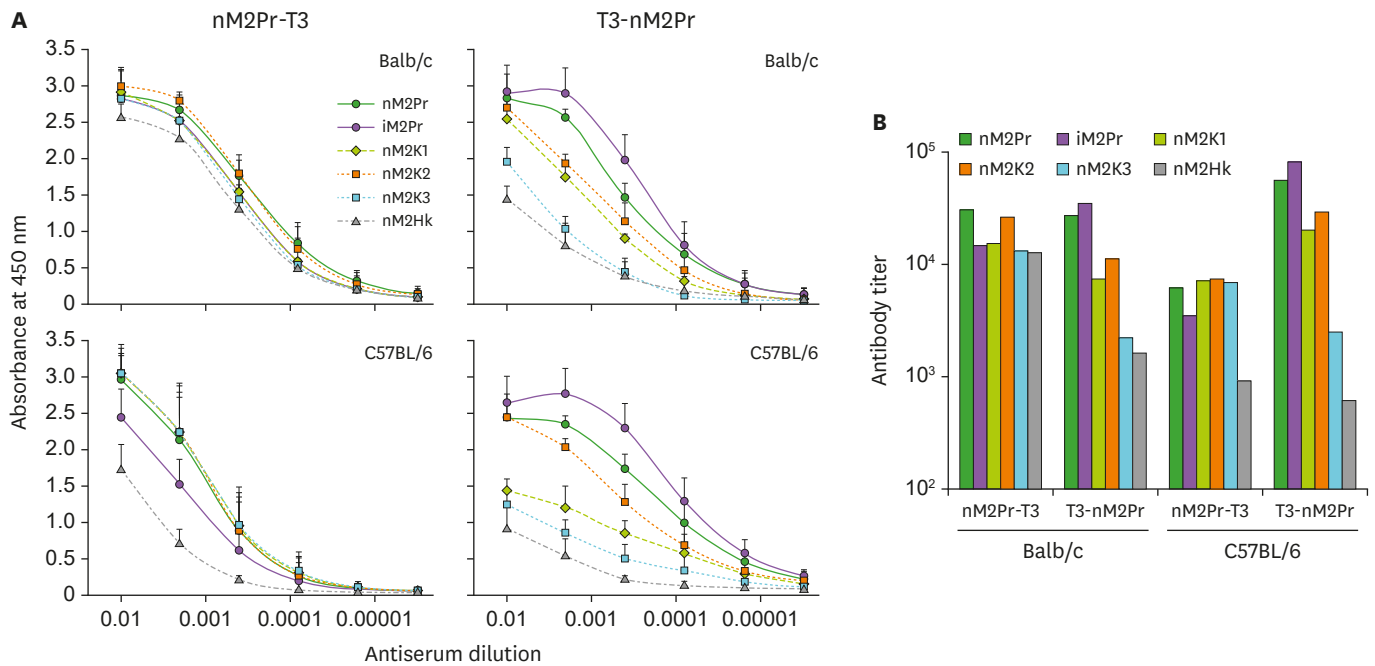


Figure 2. Reactivity of anti-nM2Pr Abs to various nM2e peptide Ags. ELISA profiles (A) and titers (B) of the Abs generated by immunization of either nM2Pr-T3 or T3-nM2Pr into the BALB/c or C57BL/6 mice. The nM2e peptide Ags conjugated to BSA were used as the coating Ags. The Ab titers are obtained by estimating the antiserum dilution when the absorbance is 0.5. The sequences of peptide immunogens and Ags are represented in **Table 1**.

respectively. All the negative control mice with PBS injection were dead within 8 days after virus challenge, while 100% and 75% of the immunized mice with T3-nM2Pr and nM2Pr-T3, respectively, were survived. The #2 dead mouse with the nM2Pr-T3 immunization showed the similar Ab titer to the nM2Pr-BSA with the #1 mouse but significantly less titer to the iM2Pr-BSA than the #1 mouse (**Fig. 3A**), suggesting that production of Abs recognizing the internal nM2Pr sequence is critical for their neutralizing activity. The results suggest that 1) the 15-mer nM2e is sufficient to induce the virus neutralization in mice and 2) the internal nM2Pr sequence recognition of anti-nM2e Abs plays an important role in their neutralizing activity for the infected PR8 virus.

In summary, we have demonstrated Ab production and neutralizing efficacy of the peptide vaccines with a 15-mer nM2e. All the nM2Pr-ThE and ThE-nM2Pr vaccines generates the anti-nM2Pr Abs recognizing the internal sequence regardless of ThEs and localization in BALB/c mice, while only T3 was relatively effective to generate Abs in C57BL/6 mice (**Fig. 1**). These result demonstrates that the ThE function is affected by the mouse MHC type. Furthermore, T3-nM2Pr more efficiently generated the Abs than nM2Pr-T3, indicates that the ThE arrangement also affects its function in C57BL/6 mice. However, the Abs against T3-nM2Pr showed the IFAV strain-specific recognizing patterns (**Fig. 2**). The Abs against nM2Pr-T3 or T3-nM2Pr are considered to recognize predominantly the common N-terminus or relatively variable C-terminus of nM2Pr, respectively, demonstrating that the nM2Pr-ThE arrangement is more effective to develop the universal IFAV vaccines. The nM2Pr immunized mice were protected from the lethal challenge of PR8 virus in C57BL/6 mice (**Fig. 3**), demonstrating that the nM2e is sufficient for protective immunity. We expect that the short nM2e-based vaccines are more beneficial for developing the M2e-based vaccines than the whole M2e, since it is more conserved and easier quality control during storage.

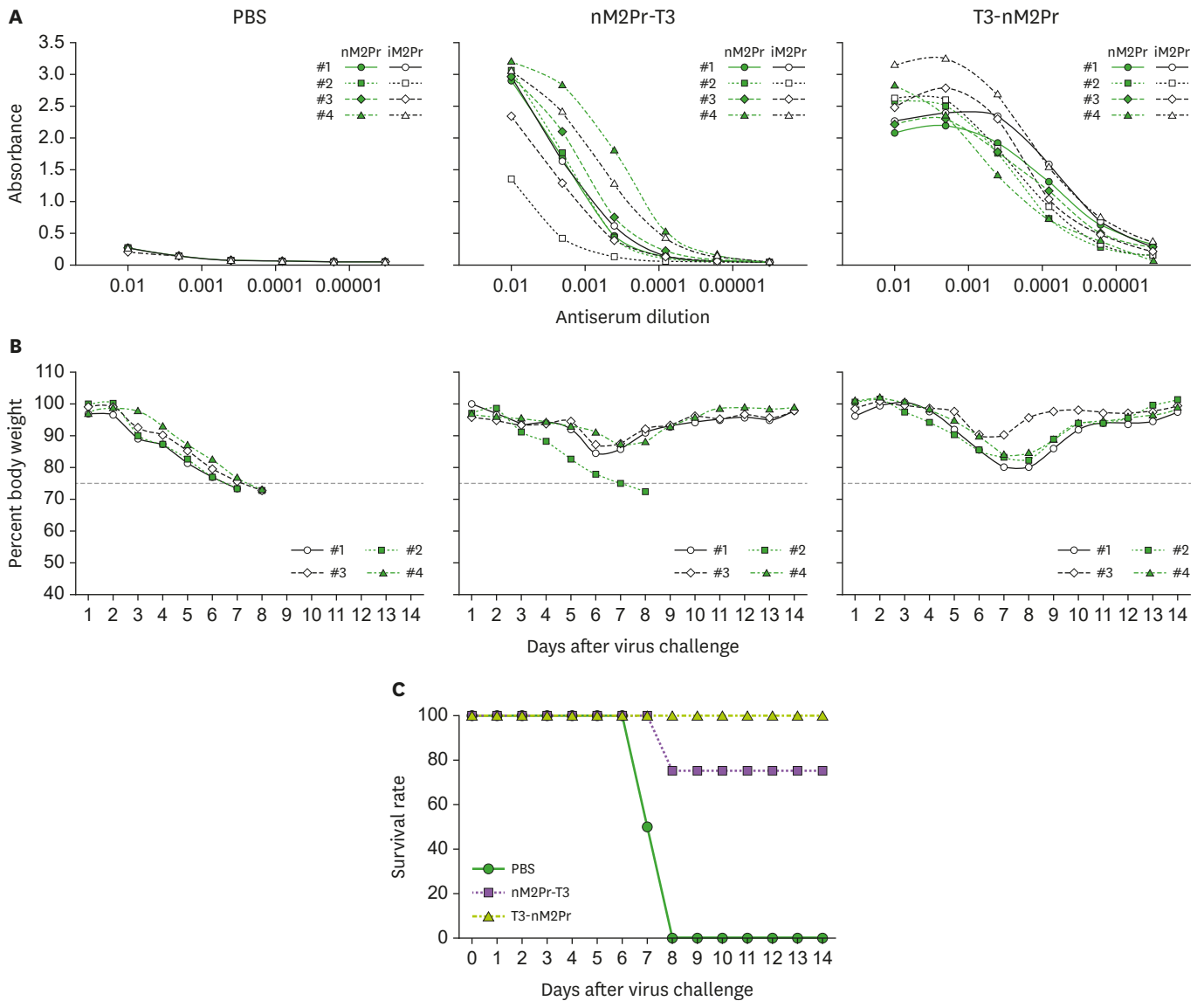


Figure 3. Protective immunity of the nM2Pr-T3 or T3-nM2Pr immunized C57BL/6 mice from PR8 virus challenge. The PBS immunized mice were used as a negative control. (A) Ab production of each immunized mouse was analyzed by indirect ELISA against nM2Pr-BSA (closed) and iM2Pr-BSA (open). (B) The immunized mice were intranasally inoculated 30 μ l of $10^{5.62}$ EID₅₀/ml of PR8 virus in PBS, and the weights of the mice were daily monitored for 14 days. (C) The survival rate of each group was judged by measuring the weight loss. The mice lost over 25% of their weight were judged to have died. #1 to #4 in each group represent individual mouse numbers.

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