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Enhanced protective immunity against H5N1 influenza virus challenge by vaccination with DNA expressing a chimeric hemagglutinin in combination with an MHC class I-restricted epitope of nucleoprotein in mice

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ABSTRACT

DNA vaccination is an effective means of eliciting both humoral and cellular immune responses. The hemagglutinin (HA) surface protein of influenza A virus is a major target of protective antibody responses induced by virus infection or by vaccination and is widely considered to be the antigen of choice for an influenza vaccine. Cytotoxic T lymphocyte (CTL) responses directed against the conserved nucleoprotein (NP) are thought to play an important role in clearing virus and promoting survival and recovery from influenza. In this study, we developed a novel DNA vaccine approach using a chimeric plasmid consisting of the HA of H5N1 influenza virus in which an MHC class I-restricted NP-specific CTL epitope (NP147–155) was inserted. Immunogenicity and antiviral efficacy of this vaccine was assessed in mouse models. A similar level of HA expression was achieved in 293T cells transfected with pHA/$NP_{147-155}$ compared to that with pHA. Besides eliciting the specific anti-HA antibody responses, vaccination using pHA/$NP_{147-155}$ in mice induced NP epitope-specific $CD8^+$ T cell responses, which are generally not inducible by vaccination with pHA alone. After H5N1 influenza virus challenge, BALB/c mice vaccinated with pHA/$NP_{147-155}$ exhibited reduced inflammation severity and lung viral titers compared to those vaccinated with pHA. Our work may contribute to improvement of HA-based influenza DNA vaccines.

1. Introduction

Influenza A virus infection continues to pose a major public health threat to humans and animals. H5N1 influenza A virus was first reported to be transmitted from poultry to humans in Hong Kong, resulting in 18 infected people and six deaths in 1997 (Claas et al., 1998). The outbreak of H5N1 influenza in humans (Subbarao et al., 1998; Yuen et al., 1998) caused wide uneasiness because humans lack immunity against this highly pathogenic influenza virus. Vaccination seems to be the most cost-effective measure for reducing the impact of influenza to humans (Palese and Garcia-Sastre, 2002). The first line of defense against influenza virus infection consists of neutralizing antibodies. Resistance to influenza was identified to correlate with serum anti-HA antibody levels and passive transfer of immune serum provides protection against subsequent influenza virus infection (Couch and Kasel, 1983; Hobson et al., 1972; Virelizier, 1975). Cytotoxic T lymphocyte (CTL)-mediated cellular immune responses also play a major role in clearing virus and promoting survival and recovery from influenza. It has been shown that the nucleoprotein (NP) of influenza virus is a major target for CTL response (Andrew et al., 1986; Epstein et al., 2002; Jeon et al., 2002; Mackenzie et al., 1989; Ohba et al., 2007; Yewdell et al., 1985). The conserved NP147–155 ($H\text{-}2^d$-restricted) epitope induced $CD8^+$ T cell responses and accelerated viral clearance in BALB/c (Bodmer et al., 1988; Crowe et al., 2003; Deliyannis et al., 2002).

Conventional influenza vaccines are produced using large stocks of vaccine viruses as materials. The process is generally cumbersome, lengthy, and costly. Most important, the resultant influenza vaccines, usually consisting of either inactivated viruses or soluble proteins, are inefficient at inducing CTL responses. As a novel vaccine candidate, DNA vaccines have been proven to induce effective antibody and CTL responses in animal models (Donnelly et al., 1995, 1997; Raz et al., 1994). Many studies demonstrated that HA-based DNA vaccines could provide protective immunity against influenza virus infection (Justewicz and Webster, 1996; Kodihalli et al., 1997, 1999; Sharpe et al., 2007). Immunogenicity of HA-based DNA vaccines was significantly improved by using codon-optimized HA sequences (Jiang et al., 2007; Wang et al., 2006). Nucleoprotein (NP) targeted DNA vaccines were also proven to be able to induce effective cross-protective immunity in animals (Bot et al., 1996; Epstein et al., 2002, 2005; Luo et al., 2008; Ulmer et al., 1993). Cloned CTLs specific for NP resulted in reduced pulmonary viral replication (Moskophidis and Kioussis, 1998).

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Table 1
The primers for amplification of the chimeric and the wild type HA fragments.

The primers for amplification of the chimeric HA/NP147–155
P1A (+) 5′-GTGAAGCTTGGGTATAATCTGTCAAAATGG-3′
P1A (−) 5′-CATTGCTTCCATGTTCTCATTTGAAGCGTCACACATTGGGTTTCCGA-3′
P1B (+) 5′-GCTTCAAATGAGAACATGGAAGCAATGTGGTCTTACATAGTGGAG-3′
P1B (−) 5′-CACTCGAGCCGGGTTATTAGTAGAAACAAGGGT-3′
P1C (+) 5′-GTGAAGCTTGGGTATAATCTGTCAAAATGG-3′
P1C (−) 5′-CACTCGAGCCGGGTTATTAGTAGAAACAAGGGT-3′
The primers for amplification of the wild type HA
P2 (+) 5′-GTGAAGCTTGGGTATAATCTGTCAAAATGG-3′
P2 (−) 5′-CACTCGAGCCGGGTTATTAGTAGAAACAAGGGT-3′

In the present study, we developed a novel DNA vaccine using a chimeric plasmid consisting of the HA of H5N1 influenza virus in which an NP specific CTL epitope (NP147–155) was inserted. Both humoral and CTL responses induced by this vaccine were examined. Antiviral efficacy against H5N1 influenza virus infection in vaccinated mice was also evaluated. These works provide a new approach for improving the protective efficacy of DNA vaccines against H5N1 influenza viruses.

2. Materials and methods

2.1. Mice and viruses

Six-week-old female BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were used for immunization and challenge studies. All mice were maintained with free access to sterile food and water.

The H5N1 avian influenza virus strain used in this study was A/chicken/Hubei/489/2004 (A/Hubei/489) (H5N1) which was mouse-adapted by passage through mouse lungs and kindly provided by Dr. Deyin Guo (Modern Virology Research Center, Wuhan, China). Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs for 48 h at 37 °C. Fifty percent egg infectious dose (EID_{50}) titers were determined by serial titration in eggs and calculated by the method of Reed–Muench. All experiments with infectious H5N1 influenza virus were conducted under BSL-3 containment, including work in animals.

2.2. Synthetic peptides

The synthetic peptide TYQRTRALV corresponding to amino acid residues 147–155 of the NP protein of the H5N1 influenza virus was purchased from HD Biosciences (Shanghai, China) at a grade of greater than 95% purity. The peptide is considered the dominant CD8 T cell determinant recognized by BALB/c mice (*H-2*^{d}) (Falk et al., 1991; Fu et al., 1997) and is common to a plurality of different influenza A virus strains (Bodmer et al., 1988; Sherman et al., 1992). The peptide was dissolved in DMEM medium for use in subsequent assays.

2.3. Construction of recombinant DNA plasmids

The plasmid pMD-HA encoding the HA gene (GenBank Accession# AY770079), which is derived from the avian influenza virus A/Hubei/489 (H5N1) strain, was used as a PCR template. A eukaryotic expression vector, pVAX1 (Invitrogen, Carlsbad, CA, USA) was used to construct DNA vaccines. The chimeric HA DNA vaccine, $pHA/NP_{147–155}$, was constructed by replacing the 7-amino acid peptide (corresponding to amino acid residues 85–91) in site E of the HA with an *H-2*^{d}-restricted immunodominant epitope of the NP protein, NP147–155 (Falk et al., 1991; Fu et al., 1997). The chimeric HA gene fragment containing the DNA sequence encoding NP147–155 was amplified by overlap PCR from the pMD-HA template using the primers P1 (A+/−, B+/−, C+/−) in Table 1. The PCR product was digested with *HindIII* and *XhoI* and was then cloned into pVAX1 to create $pHA/NP_{147–155}$. Similarly, the wild-type HA gene was amplified by PCR from the pMD-HA template using the primers P2 (+/−) in Table 1, and was cloned into pVAX1 to generate pHA. Two constructs were sequenced to confirm cloning accuracy.

2.4. Western blot analysis of in vitro-expressed wild-type and chimeric HA antigens

To confirm expression of both wild-type and chimeric HA proteins, 4 μg of each plasmid was transfected into HEK 293T cells in six-well plates (Nunc, Roskilde, Denmark) using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instruction. At 48 h post-transfection, the cells were lysed with 0.1 M Tris–HCl (pH 7.8) and 0.125% Nonidet P-40. The lysates were mixed with an equal volume of 2× SDS loading buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue and 10% β-mercaptoethanol), boiled for 10 min, subjected to 10% SDS–PAGE and transferred to nitrocellulose membranes. The expressed proteins were probed by anti-HA rabbit serum, which was prepared by our laboratory. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Pierce, Rockville, Maryland, USA) was used as the secondary antibody. The reaction was detected by DAB reagents (Amersham Bioscience, Piscataway, NJ, USA) and band intensities were analyzed by Gene-Tools from Syngene's 2D gel imaging system (Syngene, Cambridge, UK).

2.5. A growth curve of A/Hubei/489 (H5N1) influenza virus in the lung of BALB/c mice

BALB/c mice were infected with 10^3 EID_{50} influenza A/Hubei/489 (H5N1) virus in 50 μl PBS via the intranasal (i.n.) route and were sacrificed daily from 2 to 8 days post-infection (five mice for each time point). The whole lung was collected and homogenized in 1 ml PBS. Lung viral titers were determined as described in Section 2.1.

2.6. Immunizations and challenges

All DNA plasmids were purified using Qiagen columns (endotoxin free) for immunization. Six-week-old female mice (BALB/c or C57BL/6 dependent on experiment design) were vaccinated three times via the intramuscular (i.m.) route with 100 μg of DNA construct on days 0, 14 and 28, respectively. Negative control mice were vaccinated with the pVAX1 vector. Serum samples were taken 14 days after the last immunization and the mice were sacrificed. Splenocytes isolated from vaccinated mice were used for ELISPOT and intracellular cytokine staining (ICCS) assays.

For challenge experiments, 14 days after the last immunization, 10 mice per group were infected via the intranasal route using 10^3 EID_{50} of A/Hubei/489 (H5N1) in 50 μl PBS. These mice were kept under daily observation for 7 days and were then sacrificed. Whole lungs were collected for histopathologic analysis (five mice for each group) and virus titration (five mice for each group).

For histopathologic analyses, the mouse lungs were weighed individually, and were fixed with 10% formaldehyde. Lung tissues were embedded in paraffin and were cut into 5 μm sections. The tissue sections were mounted on glass slides stained with hematoxylin and eosin (H&E), and were then reviewed microscopically for histopathologic changes. The lung inflammation severity scores were defined as 0–4 as previously described (Jin et al., 2007). For virus titration, the whole lung was homogenized in 1 ml PBS and clarified homogenate was determined in eggs as described in Section 2.1.

2.7. Enzyme-linked immunosorbent assay (ELISA)

H5N1 HA-specific IgG antibody was detected by ELISA as described previously (Wang et al., 2006). Briefly, 96-well microtiter plates were coated with 100 μl/well (5 μg/ml) of the recombinant influenza HA, incubated overnight at 4 °C and washed with PBS containing 0.05% Tween-20 (PBST). Subsequently, the plates were blocked with 100 μl/well of blocking buffer (3% BSA in PBST) for 1 h at 37 °C. After five washes, 100 μl of serially diluted mouse sera was added to the triplicate wells and incubated for 1 h at 37 °C. After another set of washes, the plates were incubated for 1 h at 37 °C with 100 μl/well of horseradish-peroxidase (HRP)-labeled anti-mouse IgG (Pierce) diluted 1:1000 in PBST. Following five washes, the plates were incubated for 15–45 min with 0.1 ml of 0.045% H_2O_2 and 0.4 mg/ml of o-phenylenediamine dihydrochloride (OPD) in phosphate–citrate buffer (0.1 M citric acid, 0.2 M sodium phosphate dibasic, pH 5.0). The reaction was stopped by adding 50 μl of 2 M H_2SO_4. Absorbance at 490 nm was recorded using an ELISA reader (MULTISKAN MK3, Thermo, USA). The end titer was defined as the highest serum dilution that had an optical density reading more than twice that of the negative control serum.

2.8. Hemagglutination inhibiton (HI) assay

The HI assay was performed as described previously (Webster et al., 2002). Briefly, 25 μl (320 HA units/ml) of inactive influenza A/Hubei/489 (H5N1) was mixed with 25 μl of twofold dilutions of the specific receptor-destroying enzyme (RDE)-treated serum in PBS in V-bottom 96-well plates. After 30 min of incubation at room temperature, 50 μl of 1% chicken erythrocytes was added to each well. After mixing, the plates were kept at room temperature for 30–60 min. The HI titer was defined as the highest serum dilution that had complete hemagglutination inhibiton and all HI assays were done in duplicate.

2.9. IFN-γ ELISPOT assay

The mouse IFN-γ ELISPOT kit (U-Cytech biosciences, Utrecht, Netherlands) was used to determine the relative number of IFN-γ-expressing T cells in the single-cell spleen suspensions following the manufacturer's instruction. In brief, Nunc MaxiSorp 96-well ELISPOT plates were coated with 50 μl/well (10 μg/ml) of coating antibodies in PBS and incubated overnight at 4 °C. After several washes with PBST, the plates were blocked with 200 μl/well of PBS containing 10% blocking stock solution B for 1 h at 37 °C. Splenocytes (5×10^5) isolated from mice were added to each well in triplicate, and were stimulated with or without 100 μl/well (5 μg/ml) of influenza virus NP147–155 peptide (TYQRTRALV) for 24 h at 37 °C. Phytohemagglutinin (PHA, 5 μg/ml, Sigma–Aldrich, St. Louis, Missouri, USA) was used as a positive control. After incubation, the cells were removed and incubated with 100 μl/well (0.5 μg/ml) of biotinylated detector antibody (anti-mouse IFN-γ) for 1 h at 37 °C. The plates were washed and 50 μl/well of 2% Φ-labeled anti-biotin antibody (GABA) was added and incubated for 1 h at 37 °C (The symbol "Φ" stands for gold particles attached to goat anti-biotin antibodies). Finally, the plate was treated with 30 μl/well of Activator I/II at 37 °C for 15–45 min, and the reaction was terminated by adding distilled water. The number of spots was counted using a computer-assisted video image analyzer. The results were expressed as spot-forming cells (SFC) per million cells. A total number of five mice in each group were used for the assays.

2.10. Intracellular cytokine staining (ICCS) assay

IFN-γ-secreting $CD8^+$ T cells were detected using the protocol recommended by the manufacturer (eBioscience, San Diego, CA, USA). Briefly, splenocytes were stimulated with 100 μl/well (20 μg/ml) of influenza virus NP147–155 peptide (TYQRTRALV) for 24 h at 37 °C, and monensin was added to a final concentration of 2 μM 2 h before the end of the incubation. The cells were washed with washing buffer (3% FBS in PBS) and blocked with purified anti-mouse CD16/32 antibody (0.5 μg per million cells) and stained with a phycoerythrin-Cy5 (PE-Cy5)-conjugated anti-mouse $CD8^+$ antibody (Ly-2, 0.125 μg per million cells). The cells were fixed with 100 μl of fixation solution in the dark at room temperature for 20 min, and then permeabilized with 1 ml of permeabilization buffer, and finally stained using anti-mouse IFN-γ antibody conjugated with phycoerythrin (PE) (0.125 μg per million cells) in the dark at room temperature for 20 min followed by flow cytometric analysis.

2.11. Statistical analysis

Statistical analysis of the experimental and control data was performed using the analysis of variance (ANOVA). A p-value of less than 0.05 was considered significant. A non-parametric test was used to evaluate the lung histopathology scores of the experimental and control groups.

3. Results

3.1. Expression of the HA proteins

To examine the impact of the NP CTL epitope on priming HA-specific immune responses against influenza virus, both the chimeric and wild-type HA genes were cloned individually into the eukaryotic expression vector pVAX1 to generate DNA constructs, pHA/$NP_{147-155}$ and pHA, respectively (Fig. 1A). To confirm the expression of the HA protein, each DNA construct was separately transfected into 293T cells. Protein expression was determined by western blot. As shown in Fig. 1B, both pHA/$NP_{147-155}$ and pHA vaccines resulted in good expression of HA proteins and the HA expression levels from two constructs were similar in cell lysates as analyzed by the GeneTools software. These results indicated that replacement of the 7-amino acid residues in site E of HA with the NP147–155 peptide seems not to alter the expression level of the HA protein *in vitro*.

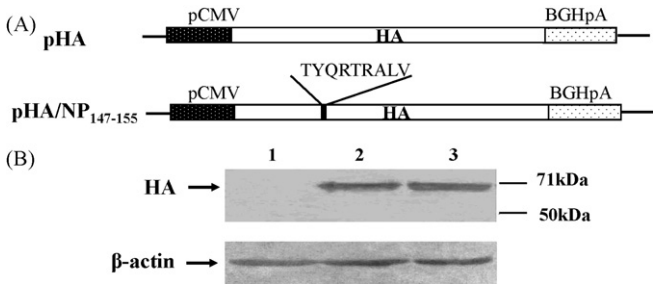

Fig. 1. (A) Schematic diagram of H5N1 wild-type and chimeric HA in the pVAX1 vector. The gene encoding a wt HA or a chimeric HA combined with an NP specific CTL epitope (NP147–155, an *H-2*^{d}-restricted dominant epitope) was cloned into the pVAX1 vector between the hCMV promoter and the BGH polyA signal, respectively. The chimeric HA form is created by replacing the 7-amino acid peptide (corresponding to amino acid residues 85–91) in site E of the HA with the MHC class I-restricted epitope NP147–155. (B) Western blot analysis of the HA expression from pHA (lane 2) and pHA/$NP_{147-155}$ (lane 3) vaccines in lysates of transiently transfected 293T cells. Lysate from cells transfected with the pVAX1 vector was used as a negative control (lane 1).

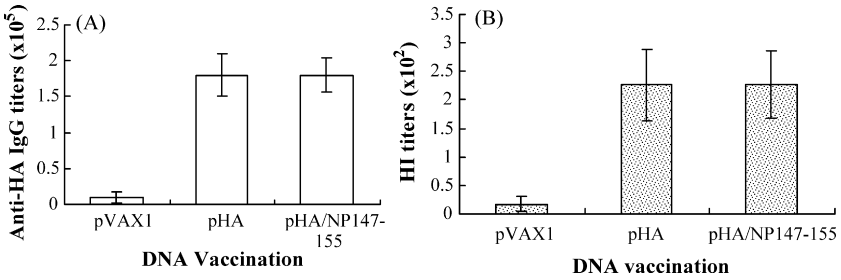

Fig. 2. Humoral immune responses in vaccinated mice. (A) Total anti-HA IgG titers determined by ELISA. Anti-HA IgG titers were expressed as the highest serum dilution that yielded an optical density greater than twice the mean of that of similarly diluted negative control samples. Data shown are mean antibody titers of five mice in each group with coefficients of variation (error bars). (B) Serum HI antibody responses in vaccinated mice. The HI titers were defined as the highest serum dilution that completely inhibited hemagglutination. Data shown are the mean HI titers of five mice in each group with standard deviations (error bars). In both ELISA and HI assays, the difference in titer between experimental groups (pHA and pHA/$NP_{147-155}$ groups) and the negative control group (pVAX1) is statistically significant ($p < 0.001$, one-way ANOVA). However, the difference between pHA and pHA/$NP_{147-155}$ groups is statistically non-significant.

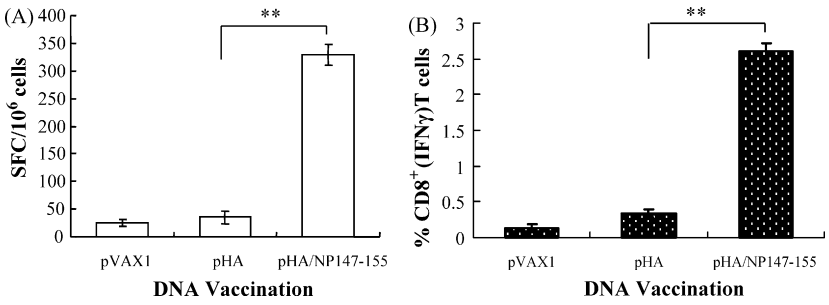

Fig. 3. Cellular immune responses in vaccinated mice. (A) In each group, five BALB/c mice were vaccinated three times with the indicated plasmid by intramuscular injection at 2-week intervals. Fourteen days after the last immunization, splenocytes isolated from the mice were collected and used for both IFN-γ ELISPOT and intracellular cytokine staining (ICCS) assays. In both assays, the difference between the pHA and pHA/$NP_{147-155}$ groups is statistically significant (**$p < 0.001$, one-way ANOVA). The data were represented as the mean ± S.D. of five mice in each group. (A) IFN-γ ELISPOT assay, and (B) percentage of influenza epitope-specific IFN-γ producing $CD8^+$ T cells in vaccinated mice.

3.2. Humoral response of vaccinated BALB/c mice

To assess humoral responses induced by these DNA vaccines in BALB/c mice, mouse sera were collected 14 days after the last immunization to measure antibody titers. As shown in Fig. 2A, immunization with pHA/$NP_{147-155}$ or pHA elicited effective HA specific antibody response in mice. There is no significant difference in the HA specific IgG antibody levels between mice vaccinated with pHA/$NP_{147-155}$ and pHA. Functional activities of the mouse sera were further investigated by determining the HI antibody titers against H5N1 influenza virus. As expected, both mice vaccinated with pHA/$NP_{147-155}$ and pHA developed similar HI antibody levels in sera (Fig. 2B).

3.3. Induction of IFN-γ secreting cells and CTL responses in vaccinated BALB/c mice

To evaluate specific T cell immune responses, splenocytes isolated from vaccinated BALB/c mice were stimulated *in vitro* with an *H-2*^{d}-restricted CTL peptide (TYQRTRALV) of NP (Bodmer et al., 1988). Mice vaccinated with pHA/$NP_{147-155}$ exhibited a strong IFN-γ producing T cell response, but pHA-vaccinated mice did not (Fig. 3A), indicating that IFN-γ producing T cells were efficiently induced and expanded in mice vaccinated with pHA/$NP_{147-155}$. To further analyze $CD8^+$ T cell-specific immunogenicities of pHA/$NP_{147-155}$, ICCS assays were performed. Splenocytes isolated from vaccinated mice were stimulated *in vitro* with the NP147–155 peptide (TYQRTRALV) for 24 h at 37 °C. The numbers of $CD8^+$ IFN-γ secreting cells were detected by staining with anti-CD8-PE-Cy5 and anti-IFN-γ-PE antibodies. As shown in Fig. 3B, mice vaccinated with pHA/$NP_{147-155}$ were found to induce about 5-fold more $CD8^+$ IFN-γ secreting cells than mice vaccinated with pHA ($p < 0.05$). These results suggested that the NP CTL determinant inserted in the chimeric HA was appropriately processed and presented *in vivo*.

3.4. Virus growth curve of H5N1 influenza virus in BALB/c mice

H5N1 influenza viruses isolated from human are known to replicate efficiently in BALB/c mice (Lu et al., 1999; Maines et al., 2005). To assess characterization of the H5N1 influenza virus (A/Hubei/489) strain isolated from chicken, the kinetics of virus propagation and morbidity were determined in BALB/c

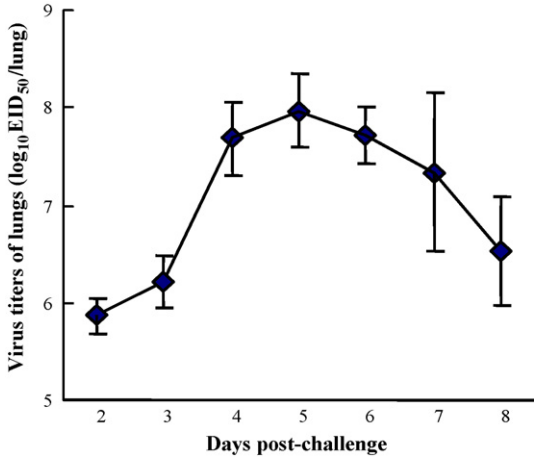

Fig. 4. Lung viral titers during infection with the H5N1 influenza virus in BALB/c mice. BALB/c mice were infected by the intranasal (i.n.) route using 10^3 EID_{50} of the H5N1 avian influenza A/Hubei/489 strain in 50 μl of PBS. Lung viral titers (represented by $\log_{10}$ EID_{50}) were determined by serial titration in eggs and calculated by the method of Reed–Muench (Mean ± S.D.; $N = 5$).

Table 2
Comparison of lung weights and virus titers of vaccinated mice with the indicated plasmids after H5N1 virus challenge infection.

Mouse strains	Vaccine	Challenge with H5N1	Mean lung weight (g ± S.D.)	Mean virus titer (log_{10} EID_{50} ± S.D.)
BALB/c	pVAX1	Yes	0.304 ± 0.066¶	7.98 ± 0.39¶
	pHA	Yes	0.216 ± 0.015	4.00 ± 0.55
	$pHA/NP_{147–155}$	Yes	0.184 ± 0.023	2.04 ± 0.81*
	Unvaccinated	No	0.158 ± 0.008	ND
C57BL/B6	pVAX1	Yes	0.287 ± 0.011†	7.07 ± 0.51†
	Pha	Yes	0.266 ± 0.013	5.05 ± 1.05
	$pHA/NP_{147–155}$	Yes	0.266 ± 0.012	5.39 ± 0.51
	Unvaccinated	No	0.151 ± 0.009	ND

Seven days post-challenge vaccinated mice were sacrificed and the lungs were collected for weights (five mice per group) and virus titers (five mice per group). Virus titers were determined by serial titration in eggs and calculated by the method of Reed–Muench. ND, not done.

* $p < 0.01$ *versus* pHA.

¶ $p < 0.01$ *versus* pHA or $pHA/NP_{147–155}$.

† $p < 0.05$ *versus* pHA or $pHA/NP_{147–155}$ (one-way ANOVA).

mice. Six-week-old female BALB/c mice were infected with 10^3 EID_{50} of A/Hubei/489 (H5N1). Lungs samples were taken daily from 2 to 8 days post-infection (dpi) for virus titration (five mice for each time point). The remaining mice were kept under daily observation for 15 days for morbidity. The results showed that the infected mice survived beyond day 15 dpi and virus propagation reached a plateau on 5 dpi in BALB/c mice. Virus titers gradually declined over the following 3 days (Fig. 4) with complete resolution of virus at 15 dpi (data not shown).

3.5. *Enhanced protection against the H5N1 influenza virus challenge induced by $pHA/NP_{147–155}$ was observed in BALB/c mice, but not in C57BL/6 mice*

To investigate the impact of the co-expressed NP CTL epitope in the chimeric HA protein on specific immune protection against the H5N1 influenza virus, BALB/c mice were infected intranasally with 10^3 EID_{50} of the H5N1 influenza virus A/Hubei/489 strain 14 days after the last immunization. Mice vaccinated with pHA or $pHA/NP_{147–155}$ had significantly reduced lung viral titers, compared with control mice vaccinated with the pVAX1vector. Importantly, lung viral titers of mice vaccinated with $pHA/NP_{147–155}$ were 100-fold less than those of mice vaccinated with pHA (Table 2), indicating that DNA vaccination with HA co-expressing an NP $CD8^+$ T cell epitope was more effective in inhibition of viral propagation and clearance of pulmonary influenza virus following challenge infection in BALB/c mice.

The histopathologic change of lung tissues from BALB/c mice vaccinated with pVAX1, pHA and $pHA/NP_{147–155}$ were also evaluated. Seven days post-challenge, mice inoculated with the pVAX1 vector had severe lung pathology including extensive inflammatory infiltrates and hypertrophy of the alveolar lining cells of lung tissues (Fig. 5A). In contrast, the lung tissues of mice vaccinated with pHA (Fig. 5B) or $pHA/NP_{147–155}$ (Fig. 5C) displayed less severe lung pathology. The average inflammation severity scores of mice vaccinated with pVAX1, pHA and $pHA/NP_{147–155}$ are about 3.8,

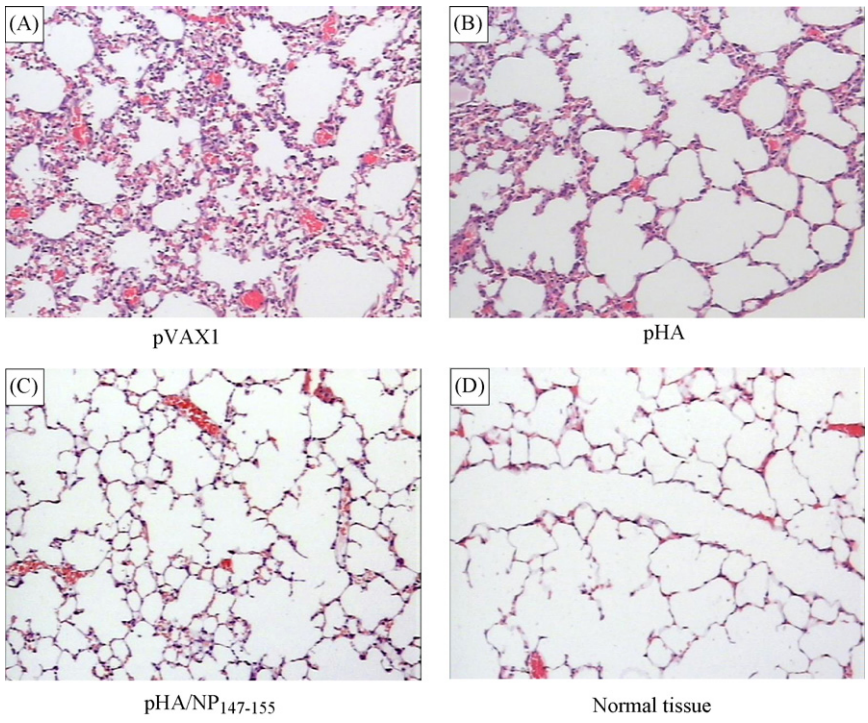

Fig. 5. Histopathologic analyses of lung tissues from vaccinated BALB/c mice. In each group, five BALB/c mice were challenged with the H5N1 avian influenza A/Hubei/489 strain 14 days after the last immunization. The mice were sacrificed 7 days post-challenge, and the lung tissues were collected for histopathologic analyses. The representative micrographs from each group are shown. Tissue sections of lungs represent mice vaccinated with pVAX1 (A), pHA (B), $pHA/NP_{147–155}$ (C), respectively, and a tissue section of the normal mouse lung for comparison (D).

Table 3
Histopathologic evaluation of lung tissues from vaccinated mice with the indicated plasmids after H5N1 virus challenge infection.

Vaccine	Number of mice per group	Number of mice with indicated lung lesion severity score					Mean severity score
		0	1	2	3	4	
pVAX1	5	0	0	0	1	4	3.8
pHA	5	0	1	2	2	0	2.2*
pHA/$NP_{147–155}$	5	1	2	2	0	0	1.2**

Seven days post-challenge vaccinated BALB/c mice were sacrificed and the lungs were collected for histological analysis. The lung inflammation severity scores were defined as 0–4 as described previously (Jin et al., 2007). 0 = not present, 1 = minimal, 2 = mild, 3 = moderate and 4 = marked.

** $p < 0.01$ *versus* pVAX1.

* $p < 0.05$ *versus* pVAX1 (a non-parametric test).

2.2 and 1.2, respectively (Table 3). Accordingly, mice vaccinated with the pVAX1 vector had significantly increased lung weights compared with mice vaccinated with pHA or pHA/$NP_{147–155}$ ($p < 0.05$) (Table 2). The lung weights of mice vaccinated with pHA/$NP_{147–155}$ were lower than those of mice vaccinated with pHA (Table 2).

To confirm whether the enhanced protection of the *H-2*^{d} dominant CD8$^+$ T cell epitope in pHA/$NP_{147–155}$ was dependent on the *H-2*^{d} haplotype of mouse strains, C57BL/6 mice (*H-2*^{b}) were used as the experimental control. C57BL/6 mice vaccinated with pHA or pHA/$NP_{147–155}$ exhibited reduced viral titers in the lungs, compared to mice vaccinated with the pVAX1 vector ($p < 0.05$) (Table 2). However, unlike in BABL/C mice, there was no significant difference in lung viral titers between pHA/$NP_{147–155}$ and pHA vaccinated C57BL/6 mice. Similarly, there was no significant difference in total lung weights between C57BL/6 mice vaccinated with pHA/$NP_{147–155}$ or with pHA (Table 2). These results indicated that the enhanced protection against H5N1 influenza virus by pHA/$NP_{147–155}$ vaccination was dependent on the recognition of the *H-2*^{d} dominant CD8$^+$ T cell epitope in BALB/c mice.

4. Discussion

Data presented in this report proved that construction of the chimeric HA protein combined with an NP specific CTL epitope was effective for improving the protective efficacies of influenza HA-based DNA vaccines. Several studies have explored and confirmed the efficacy of the influenza HA-based DNA vaccines, which successfully induced immune responses to influenza viruses in several different species, including chickens, swine, mice and ferrets (Larsen and Olsen, 2002; Robinson et al., 1993). Recently, many attempts have been made to further improve the efficacy of HA-based DNA vaccines (Fynan et al., 1993; Jiang et al., 2007; Kodihalli et al., 1997; Wang et al., 2006). Several studies demonstrated that immunization with combined HA and internal protein DNA plasmids was more effective than immunization with individual HA or internal protein DNA alone (Donnelly et al., 1995; Kodihalli et al., 2000; Xie et al., 2007). Immunization with an NP-based DNA vaccine also induced significant immune responses and cross-protection against influenza virus (Chen et al., 1999; Epstein et al., 2002, 2005; Luo et al., 2008; Ohba et al., 2007; Roy et al., 2007; Saha et al., 2006; Ulmer et al., 1993, 1998; Yewdell et al., 1985).

Naturally occurring immune responses do not recognize all possible epitopes, but are instead commonly focused on relatively few epitopes. The phenomena of epitope selectivity and differential prominence have been found in mice vaccinated with NP DNA and/or infected with influenza virus (Fu et al., 1997; La Gruta et al., 2006). Although both dominant and recessive epitopes of NP in vaccinated mice are presented, splenocytes from BALB/c mice infected with influenza virus exhibited a CTL response directed against only the dominant epitope (Fu et al., 1997). It has been reported that CD8$^+$ T cells played important effector roles in protective immunity against influenza virus challenge in mice vaccinated with NP DNA (Liang et al., 1994; Ulmer et al., 1998). The minigene NP147–155–VAC construct induced a greater primary pulmonary CTL response than the full-length NP–VAC recombinant (Lawson et al., 1994). Since the short peptide consisting of amino acids 147–155 of NP is an immunodominant MHC class I-restricted CD8$^+$ T cell epitope (Falk et al., 1991; Fu et al., 1997), we inserted this epitope, instead of the entire NP, into HA. Immunization with the chimeric DNA vaccine, pHA/$NP_{147–155}$, induced a high specific HA antibody level and effective NP147–155 epitope-specific CD8$^+$ T cell immune responses, as well as accelerated clearance of influenza virus infection in BALB/c mice. However, the accelerated clearance of influenza virus in lungs was not observed in C57BL/6 mice (*H-2*^{b}) (Table 2), suggesting that the enhanced protection against the H5N1 influenza virus by pHA/$NP_{147–155}$ vaccination was dependent on the recognition of the *H-2*^{d} dominant CD8$^+$ T cell epitope in mice.

Currently, the H5N1 avian influenza virus has been reported to have lethal effects on humans following direct infection from birds (Horimoto and Kawaoka, 2005; Lewis, 2006; Steinhauer, 1999). During the period from 2003 to September 2008, widespread outbreaks of H5N1 occurred in Asian, European and African countries, resulting in 387 human cases, with a total of 245 reported deaths around the world (http://www.who.int/csr/disease/avian_influenza/en/). This outbreak presents a challenge in the event of influenza pandemics where high morbidity and mortality is feared, as is possible with reassortment strains derived from the avian H5N1 viruses or with strains similar to those that have caused sporadic outbreaks in humans. Since the speed of viral dissemination or antigenic drift makes it almost impossible to produce large amounts of the vaccines using the conventional technology, in a timely fashion to meet urgent needs in the event of a pandemic, influenza DNA vaccines have attracted much attention since they were first reported to induce protective immune responses (Ada and Ramshaw, 2003; Ulmer, 2002). Previous results showed the HA DNA vaccine lacked effective immunity when given by the intramuscular (i.m.) route in the phase clinical I trial. However, its immune efficacy was importantly improved and showed promise when delivered by particle-mediated epidermal delivery (PMED) (Drape et al., 2006). In the current study, we developed a novel strategy to improve the efficacy of HA DNA vaccines in a mouse model. Our data showed that vaccination of mice with pHA/$NP_{147–155}$ induced the enhanced protection against H5N1 influenza virus infection in BALB/c mice, suggesting that it is feasible to use DNA vaccines that express multiple epitopes to fight against H5N1 influenza viruses. Because the human MHC-I locus is highly polymorphic, it is necessary to find promiscuous peptides that bind to a number of HLA alleles. Future work will focus on evaluating characterization of multi-epitope peptides in different MHC-I molecular backgrounds.

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