

Full Length Research Paper

Reconstructed H3N2 influenza virus predicted from influenza vaccine strains improved cross-protective immunity in mice

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Hemagglutinin (HA) protein of influenza virus is a core antigen protein which induces protective antibody in hosts. But HA genes mutate rapidly. Every year, World Health Organization (WHO) selects representative influenza virus strains from the influenza centers worldwide for virus vaccine production. So, the selected influenza vaccine strains, can partly respond to antigen drifts of circulating influenza virus, especially the reconstructed H3N2 which induces the cross reaction. It is reported here using the Immune Epitope Database and the reverse genetic method on how to produce reassortant influenza virus based on the changes of B and D antigenic regions (B antigenic region: 156-160aa, 187-198aa; D antigenic region: 167-182aa, 201-215aa) of HA protein of seasonal influenza H3N2 vaccine strains over twenty years. In a mouse model, the attenuated reassortant viruses induced neutralization antibodies, cross-reactive T-cell responses, and were protective against different lethal influenza virus challenge. So, through the analysis of the antigenic regions of HA using computer and software methods, the reconstructed rB/D influenza virus mostly induced cross protection in mouse model.

Key words: influenza, vaccine strains, antigen epitope, cross-protective.

INTRODUCTION

Influenza virus causes influenza epidemics, which continues to impose a significant impact on the world's population, especially resulting in human suffering and economic burden (Nair et al., 2011; Molinari et al., 2007). Currently, the major subtypes circulating in human populations are influenza A/H3N2 and A/H1N1 subtypes

(Pediatrics, 2012; Harper et al., 2009; Rambaut et al., 2008). HA is a special factor due to its role in the viral entry mechanism and immune recognition (Ge et al., 2010; Babon et al., 2012; Bean et al., 1992). It consists of two subunits, HA1 and HA2. Through continuous antigenic mutations, HA1 contains the receptor-binding

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and antigenic domains, experience a process termed positive Darwinian selection (Corti et al., 2010; Han and Marasco, 2011). The variable antigenic regions of the HA1 domain are potential targets of neutralizing antibodies. And thus, amino acids substitutions at these regions (A to E) have been associated with annual epidemics in humans (Carrat and Flahault, 2007; Wang et al., 2009; Suzuki, 2008). The influenza vaccines are used for the prevention of influenza (Centers for Disease Control and Prevention, 2013; Sook-San et al., 2013). The strain that will cause the pandemic could not be predicted (Kaminski and Lee, 2011; Wright, 2008). Therefore, development of a vaccine that induces broadly cross-protective immunity against variant viruses is urgently needed (Gomez et al., 2013).

In this study, it was discovered that some amino acids changes of B/D antigenic regions were critically important in improving IgG and IgA antibodies and T cell immunity. In addition, results showed that reassortant virus induced cross-protective immunity in mice. It was predicted that HA1 antigenic regions change in seasonal H3N2 influenza vaccine strains during the past twenty years. It was discovered that amino acid substitutions occurred mainly in B and D antigenic regions of HA protein. Through the prediction of T cell's epitopes, the relationship between amino acids changes of B and D antigenic regions and its immunogenicity and heterologous protection were investigated.

MATERIALS AND METHODS

Influenza vaccine strain (H3N2) HA amino acid sequences

The major surface analyzed was glycoprotein hemagglutinin (HA). The Influenza Research Database website and NIBSC (National Institute for Biological Standards and Control) was used, and the downloaded seasonal influenza A vaccine strains gene pregments which had whole full-length HA protein in the past twenty years (World Health Organization 2015).

Sequence analysis

Bio Edit version 7.0.9.0 was used for HA protein analysis. The four antigenic regions: A, B, C and D were compared in H3 HA molecules (Figure 2). HA proteins were compared to predict the conserved T cell epitopes. The Immune Epitope Database (IEDB) was used to predict CD4+ and CD8+ T cell epitopes of HA proteins. Human leukocyte antigen (HLA)-DRB1 alleles were selected to identify the CD4+ T cell epitopes, NetMHCIIpan (3.1 Server) was selected as prediction method. The prediction values are given in nM IC50 values and as % Rank to set of 200 random natural peptides. Threshold for strong binding peptides (IC50): 50.000 nM; Threshold for weak binding peptides (IC50): 500.000 nM; Affinity (nM) were predicted with binding affinity in nanomolar IC50.

Virus cell culture

Madin-Darby canine kidney (MDCK) (ATCC: CCL-34) and 293T human embryonic kidney cell (ATCC) were grown at 37°C in

Dulbecco's Modified Eagle Medium (DMEM) containing (1%) bovine serum albumin (BSA). DMEM/F12 (pH 7.0-7.2) containing (1%) bovine serum albumin (BSA), 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES buffer and (1%) L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin were used for virus growth. A/TaiZhou/13/2009(H3N2)(TZ13) used in this study was isolated from a patient in the Zhejiang Province of China, and was propagated in MDCK cells. This virus, termed TZ13, maintained a high titer in MDCK cells. The Influenza A/Puerto Rico/8/1934 (H1N1) virus was obtained by reverse genetics as previously described (Hoffmann and Webster 2000) using plasmids bearing the eight genes defined by accession numbers AF389115 to AF189122 in the NCBI Database.

Virus recovery

Template viral RNA was extracted from A/TaiZhou/13/2009(H3N2) using the QIAamp viral RNA extraction kit (Qiagen, Valencia, CA). The PB2, PB1, PA, NP, M, NS and NA genes of A/TaiZhou/13/2009 (H3N2) were reverse-transcribed using a reverse transcription kit (Biovisualab Ltd, Shanghai, China), the HA gene were synthetically produced according to the predicting results (Figure 2), and the HA sequence was confirmed by sequencing analyses. The cDNAs were amplified by HiFiFast DNA Polymerase (Biovisualab Ltd, Shanghai, China) using primers:

PB2 forward: 5'TATTggTCTCAgggAgCAAAAgCAggTC3',
 PB2 reverse: 5'ATATggTCTCgTATTAgTAgAAACAAGgTCgTTT3';
 PB1 forward: 5'TATTCgTCTCAgggAgCAAAAgCAggCA3',
 PB1 reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAGgCATTT3';
 PA forward: 5'TATTggTCTCAgggAgCAAAAgCAggTAC3',
 PA reverse: 5'ATATggTCTCgTATTAgTAgAAACAAGgTACTT3';
 NP forward: 5'TATTCgTCTCAgggAgCAAAAgCAgggTT3',
 NP reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAGgTATTTTT3';
 NA forward: 5'TATTggTCTCAgggAgCAAAAgCAggAgT3',
 NA reverse: 5'ATATggTCTCgTATTAgTAgAAACAAGgTTTTTT3';
 M forward: 5'TATTCgTCTCAgggAgCAAAAgCAggTAG3',
 M reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAGgTAGTTTTT3';
 NS forward: 5'TATTCgTCTCAgggAgCAAAAgCAgggTg3',
 NS reverse: 5'ATATCgTCTCgTATTAgTAgAAACAAGgTgTTTTT3'.

Viruses were generated as described previously (Hoffmann and Webster, 2000). After confirming the sequences of all inserts by sequencing, the correct plasmids were used for virus recovery. Recovery of reassortant H3N2 influenza viruses was achieved by transfecting seven plasmid DNAs of TZ13 (TZ13-PB2, TZ13-PB1, TZ13-PA, TZ13-NP, TZ13-M, TZ13-NS and TZ13-NA), and plasmid encoding the different surface antigen genes of HA (PBR322-HA) (Figure 2) into 293T cells using Lipofectamine™2000 (Invitrogen, CA). After 48 h, the transfected cells and supernatants were harvested for reassortant virus analysis. Briefly, the genes of the TZ13 virus were amplified by RT-PCR and inserted into plasmid pHW2000, virus stock were propagated in vero cell. Three reassortant influenza viruses were obtained and were termed rB, rD, rB/D influenza virus, rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions. The reassortant viruses rB, rD, rB/D were obtained using the reverse genetic method, in which the seven backbone fragments PB2, PB1, PA, NP, M, NS, and NA were derived from TZ13, whereas the HA surface antigen, B/D antigenic regions and other regions were synthesized in HA backbone fragment of TZ13.

Replication kinetics in MDCK cells

Multi cycle replication curves were generated according to inoculate

MDCK cell at a multiplicity of infection (MOI) of 0.01 and 50% tissue culture infectious doses (TCID₅₀) per cell in duplicate. After inoculation, supernatants were sampled at 6, 12, 24 and 48 h, and virus titers in these supernatants were decided by means of end-point titration in MDCK cells.

Virus infectivity titration, TCID₅₀

TCID₅₀ (50% tissue culture infectious dose) titer was determined using the MDCK cells. Cells grown to the confluence in flat bottom 96-well plates were washed with PBS, and inoculated with serial 10-fold dilutions of the virus sample (diluted in full EMEM medium without FBS). Inoculated cells were incubated at 33°C, 5% CO₂ for 72 h. Cell infection in a given well was determined by cytopathic effect. TCID₅₀ titers were calculated by the method of Reed and Muench (1938).

Pathogenicity of reassortant viruses in mice

All animal experiments were conducted in accordance with the Guidelines for Animal Experiments described and approved by the Institute of the Fujian Medical University animals. To assess the pathogenicity of reassortant viruses, groups of eight 4-week-old BALB/c were inoculated with the reassortants viruses at a standard dose 10^{8.0} TCID₅₀ by the intravenous route (i.v.) and observed for 14 days.

ELISA

The specific IgG and IgA titer against the reassortant viruses were determined by an indirect ELISA. Microtiter plates were coated with reassortant viruses (64 hemagglutinating units (HAU) per 50 ul) overnight at 4°C. Plates were washed with PBS-T buffer (PBS with 0.05% Tween 20) and blocked with (5%) skimmed milk diluted in PBS for 1 h at 37°C. Serum samples were diluted in dilution buffer (1% skimmed milk in PBS) and added to plates. Serum was two-fold serially diluted (starting from 1:10) and allowed to incubate for 2 h at 37°C. After washing in PBS-T, alkaline phosphatase-conjugated goat anti-mouse IgG, IgA (Sigma), diluted 1:1000 in PBS-T, was used as the detection antibody, with p-nitrophenylphosphate as a substrate (Sigma). The optical density (OD) was read at 405 nm using a Multiskan Ascent plate reader after substrate addition. Reciprocal serum antibody titers were calculated at 50% maximal binding on the titration curve. End point dilution titers were determined as the reciprocal dilution of the last well which had an OD₄₀₅ above the mean OD₄₀₅ plus two standard deviation of naive animal sera.

ELISPOT assays

Spleens from immunized mice were removed and single cell suspensions were prepared. Single cell suspensions of lymphocytes were suspended at 1×10⁶ cells/200 ul in RPMI 1640 supplemented with (10%) heat-inactivated fetal bovine serum. The cells were cultured in triplicate and plated in enzyme-linked immunosorbent spot (ELISPOT) plates (BD Pharmingen) that have been previously coated with IL-4 or IFN-γ capture antibody (BD Pharmingen) overnight at 4°C, and then stimulated with variant virus at an MOI of 1. The spot-forming cells were detected by addition of biotinylated IL-4 or IFN-γ detective antibody, followed by the addition of streptavidin-HRP and development with AEC substrate solution. Wells containing no antigen or 10 ug PMA were used as negative and positive control, respectively. Spots were

counted using AID Immunospot (Cellular Technology Ltd.).

Cross-protection experiment

To evaluate the immunogenicity of reassortant viruses, 4-to 6-week-old female BALB/c mice (20 in each group) were immunized with two dose (days 0 and 14) of 10^{6.0} TCID₅₀ by the intravenous route (i.v.). Mice were challenged intranasally with 20 μl containing 10^{8.0} TCID₅₀ of A/PR/8/34 and TZ13 monitored for death or survival over a period of 14 days.

Statistical and bioinformatics analyses

Data was evaluated using GraphPad Prism 5 software. The statistical significance of difference was evaluated by two-ANOVA. A p value of p<0.05 was considered significant. The nucleotide sequences determined in this study was available from GenBank under Sequin numbers DQ017486.

RESULTS

Selection of H3N2 influenza vaccine strains

Seasonal H3N2 influenza vaccine strains full-length HA protein sequences that were unrepeated from Influenza Research Database over the past twenty years (1990-2012) from the Northern hemisphere was selected. Based on the above criteria, six H3N2 influenza vaccine strains were discovered. Also, the amino acid changes in the HA proteins of the H3N2 influenza vaccine strains were compared, and it was found out that there were 54 amino acids changes in the HA proteins. The amino acids changes were concentrated on the HA1 region, which was 45/54. The HA1 region was found variable, while HA2 region was conserved over the entire evolution of the H3N2 viruses within the study period (1990 -2012).

Predicted conserved hemagglutinin CD4+ T and CD8+ T cells epitopes on H3N2 influenza vaccine strains

In order to have a better understanding of the antigenic changes of seasonal influenza vaccine strain H3N2, the gene sequence was analyzed and the HA proteins for the four antigenic regions: A,B,C,D in H3 HA molecules were compared (Figure 1). During the period of twenty years, it was discovered that amino acids changes were mainly in B/D antigenic regions. So, B/D regions were selected to predict the hemagglutinin CD4+ T and CD8+ T cells epitopes and only one group of B/D antigenic regions was decided on. The predicted epitopes of the B/D group exhibited strong MHC- I/MHC- II binding affinity. The strong binding affinity thresholds were >500 nM. The degree of predicted epitope was validated on protective immune with the experiments. Decision on T cell epitopes in highest binding affinity of the B and D antigenic regions

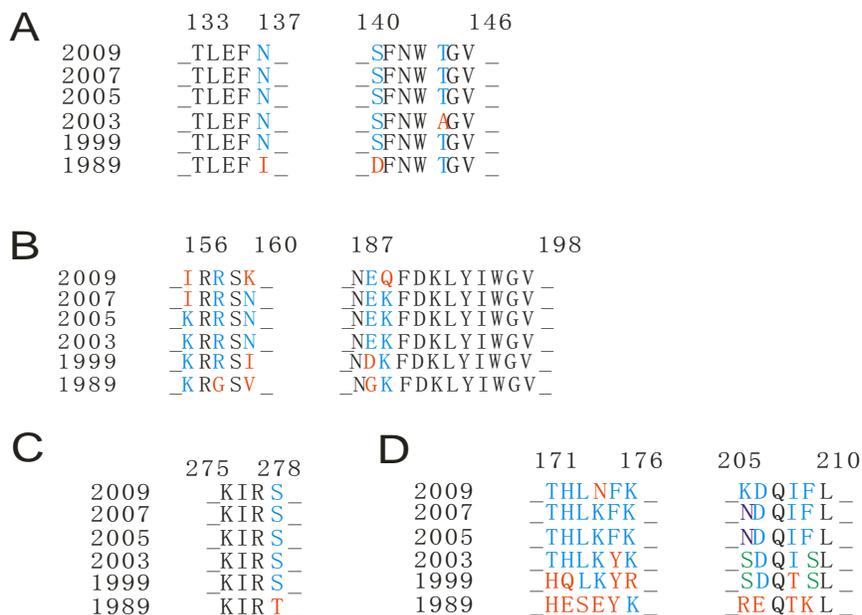


Figure 1. The analysis and comparisons of the HA protein of H3N2 seasonal influenza vaccine strains from Influenza Research Database over the past twenty years (1990-2012) from the Northern hemisphere.

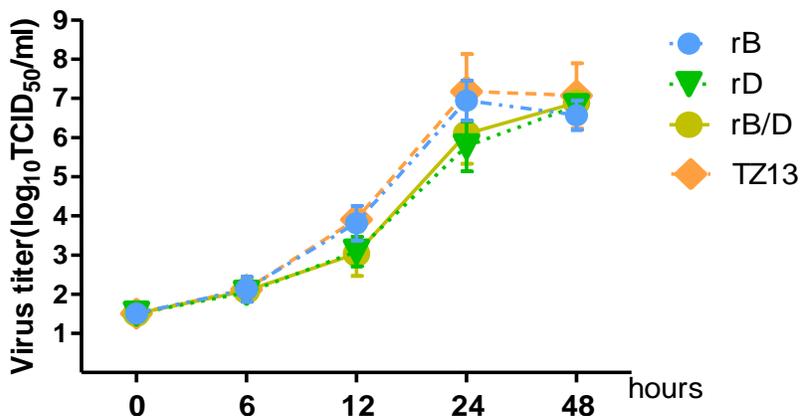


Figure 2. Growth kinetics of reassortant viruses in MDCK cells. Mean and standard deviations of three repeat assays are shown. The viral titer was detected by TCID₅₀. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

of the HA protein, and the amino acid position of B region (156-160,187-198) were KRRSN and NEQFDKLYIWGV, the amino acid position of D region (167-182, 201-215) were LNKRQHSNEKYPALNV and PGTDNDQTELYAQAS.

virus was 107.0TCID₅₀. These results show that, the growth kinetics of reassortant viruses and the reassortant viruses obtained has similar ability for growth in mammalian cells.

Growth kinetics of reassortant viruses

All the reassortant viruses replicated to a similar extent at 6, 12, 24, 48 h (Figure 2). At 48 h, the titer of reassortant

Pathogenicity of reassortant viruses in mice

To further investigate the potential effects of mutations on pathogenicity, the context of the mouse model was

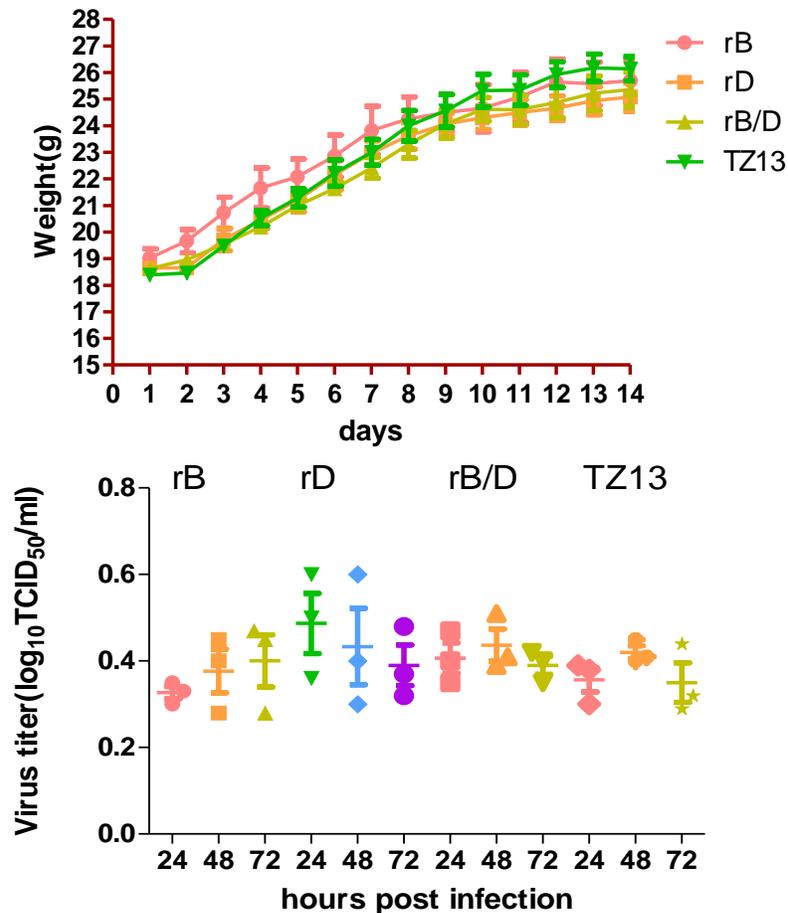


Figure 3. Changes of B/D antigenic regions in the HA protein increase weight and did not increase viral replication in mice. Mice were infected by the i.v., and weight loss (A) was monitored. (B) Lung tissue was collected from mice (n=3) at different time points after infection and homogenized, and viral titers were determined by TCID₅₀. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

characterized. Groups of eight-week-old females were inoculated at a standard dose $10^{8.0}$ TCID₅₀ by the intravenous route (i.v.) and then monitored for weight loss (Figure 3) and survival daily for 14 days to assess the effects of the infection. All mice remained healthy throughout the 14-day observation period with no mortalities. Mice weight and behavior were recorded and observed daily for 14 days as shown in Figure 3, the body weights of the four groups were very similar to that of the weight group. The weight increased from 18.3 ± 0.5 g at day 1 to 26.8 ± 1.5 g at day 14.

In a subsequent experiment, mice infected with the same amount of the respective viruses were euthanized at the indicated time points and viral titers in the lung were determined by TCID₅₀ (Figure 3). As expected, mice infected with reassortant viruses displayed low titer at all time points. These results demonstrate that reassortant viruses have a low pathogenicity similar to

that of A/nan chang.

Humoral immune responses in mice

To assess the ability of the reassortant viruses to induce virus specific immunity against influenza virus, 4- to 6-week-old female BALB/c mice (20 in each group) were immunized twice (days 0 and 14) with one dose of $10^{6.0}$ TCID₅₀ by the intravenous route (i.v.). Three weeks after the final vaccination, to examine virus-specific antibody production, IgG and IgA in the nasal wash, BAL and serum of immunized mice were measured by using ELISA (Figure 4). As shown in Figure 4, when compared with the wt group, vaccination with rB/D virus induced significantly strong antibody response in BAL and serum. Notably, both the virus-specific IgG and IgA titer in BAL, and the IgG in serum from mice immunized with rB/D

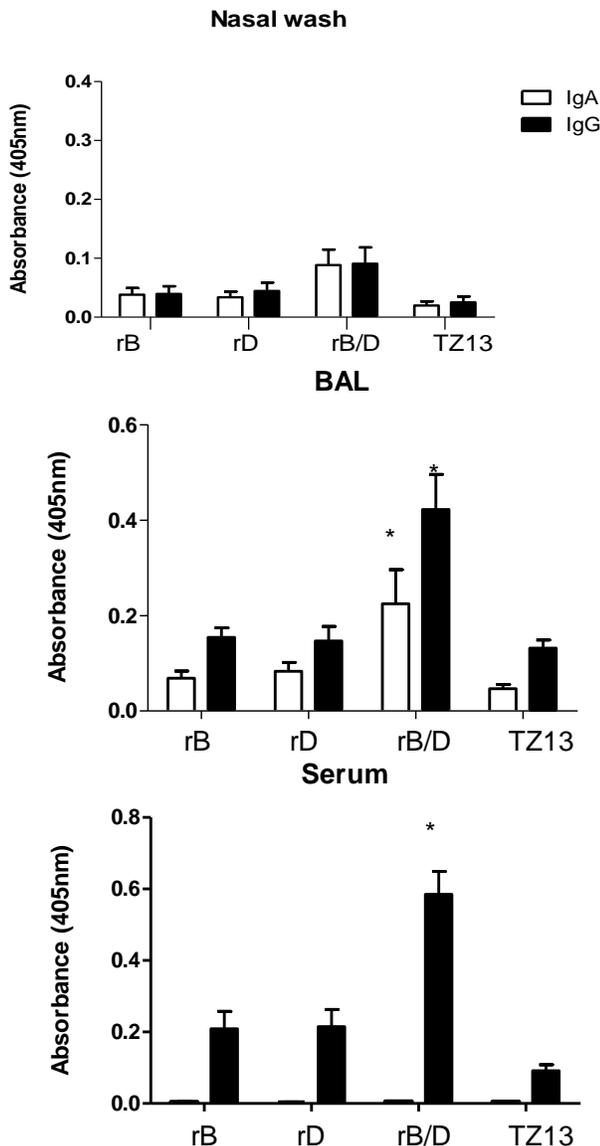


Figure 4. Induction of virus-specific IgG and IgA in nasal wash, BAL and serum of mice immunized with reassortant viruses. Virus-specific antibodies were detected by means of an ELISA. Sample (n=5) from each group were obtained 21 days after the vaccination. Results are expressed as the mean and standard deviations absorbance of 1:10 diluted samples (serum). Statistically significant differences among the groups were assessed by TWO-ANOVA (*P<0.05). rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

reassortant virus were higher than the respective titers from mice immunized with other three influenza viruses.

T helper cell responses in mice

To investigate the T helper cell responses, splenocytes

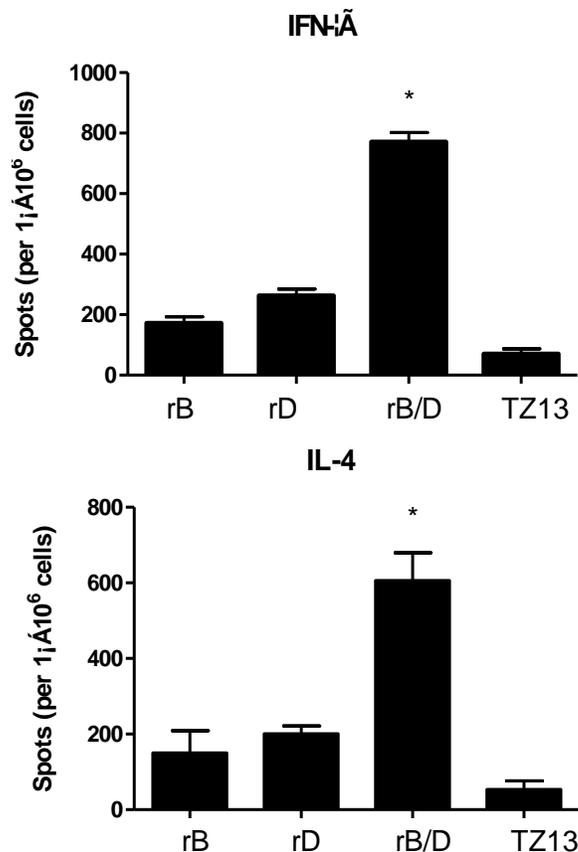


Figure 5. The level of IFN- γ and IL-4 spot-forming cells in spleens of mice as determined by ELISPOT assays. Mice were immunized and splenocytes were isolated and stimulated with reassortant viruses. Bars represent mean and standard deviations of spot counts in triplicate wells. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

were prepared on day 21 and stimulated with the reassortant viruses *in vitro*. The numbers of IFN- γ and IL-4 producing cells were determined by ELISPOT assays. The results indicated a mixed Th1 (INF- γ) and Th2 (IL-4) response was observed (Figure 5). TH-1 and TH-2 type responses were found to be predominant at rB/D group. IL-4 and INF- γ responses were relatively higher in mice immunized rB/D influenza virus. These results demonstrate that rB/D influenza virus can improve the higher immunity than other reassortant influenza viruses in mice.

Cross-protection studies in mice

BALB/c mice were used for challenge and protection studies. Two weeks after the boost immunization, all animals were challenged with the heterologous PR8. Challenge with the PR8 resulted in a transient body

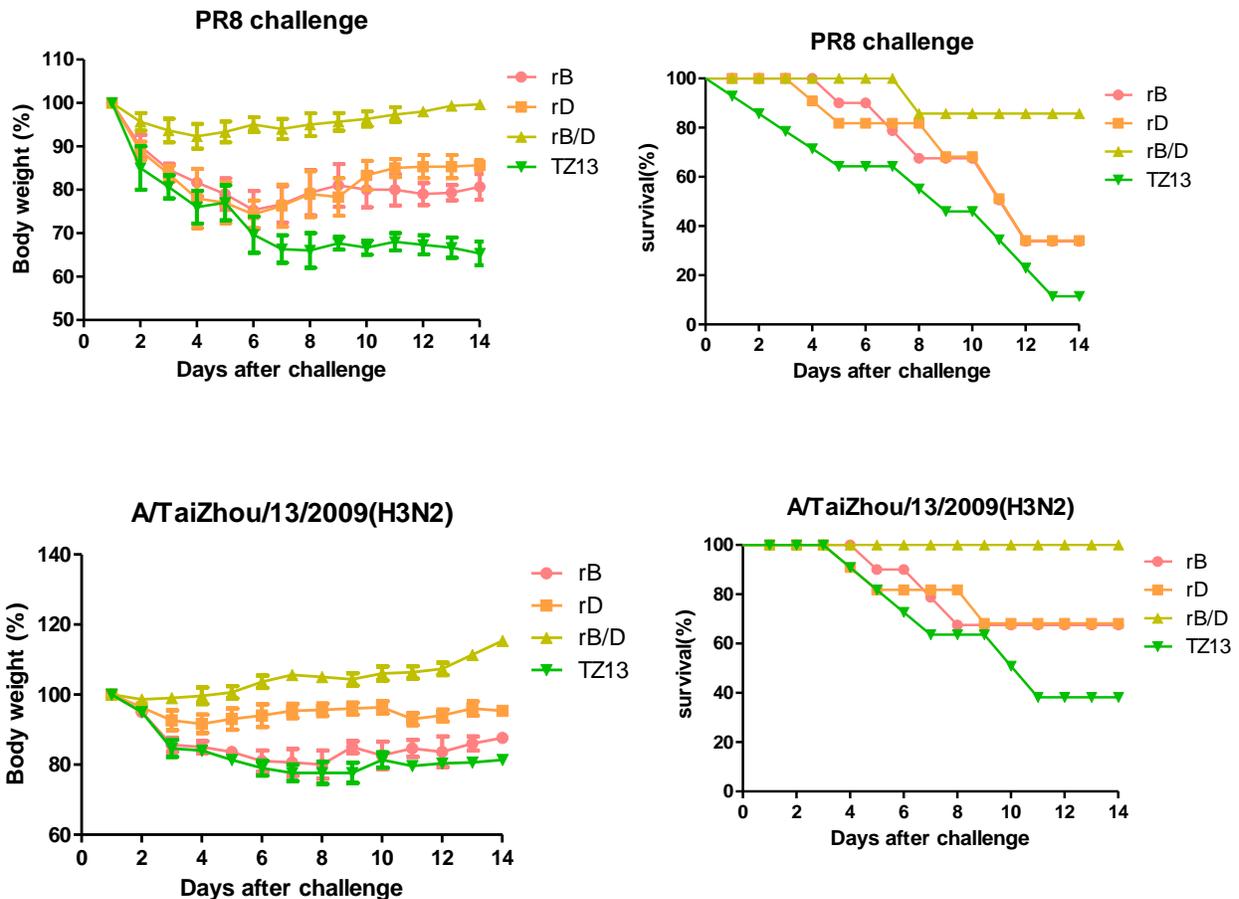


Figure 6. Body weight changes and survival rates of mice after different lethal influenza virus challenge. A body weight changes and survival rates of mice after challenge with the PR8; B body weight changes and survival rates of mice after challenge with the TZ13. Each point represents the mean of five mice on each day. rB means the change of B antigenic region, rD means the change of D antigenic region, rB/D means the change of B and D antigenic regions.

weight loss at day 7, recovery on the following 7 days, and rB/D group has the lowest body weight loss (10%) and the highest survival (80%) among reassortant influenza viruses groups, whereas the other groups, 40% were dead. There was significant difference between the rB/D group and other three groups ($P < 0.05$). These results indicated that rB/D influenza virus is the most effective to protect mice against heterologous lethal influenza virus challenge.

DISCUSSION

Influenza vaccine strains must be selected each year by WHO collaborating centers because influenza A viruses undergone sufficient antigenic drift to evade existing antibody responses (Wright et al., 2006). Influenza virus hemagglutinin is important for virus virulence, sometimes, only one amino acid substitution often interferes with virus virulence and resistance (Chen et al., 2010; Liu et al., 2010). HA protein of influenza virus is a main

component of influenza vaccine. Based on HA protein, there were amounts of influenza vaccines studies (D'Aoust et al., 2010; Steel et al., 2010). Owing to the similarity and specificity of HA protein in different types and subtypes, cross-reactive antibodies usually appear to protect against different influenza virus infections (Wrarmert et al., 2011). Although, functional antibody responses are an accepted correlate for vaccine induced protection, there are increasing reports that T-cell responses are important.

Reverse genetic technology, is used to generate influenza virus from cells co-transfected with plasmids of influenza virus gene segments, and has been applied to vaccine development since 1998. The influenza A H3N2 virus A/Taizhou/13/2009 (TZ13) was isolated from a patient in Hangzhou of China in 2009. In this study, the backbone from TZ13 was employed using reverse genetics and some reassortant viruses were generated. The HA protein segments of reassortant viruses were predicted from T cell epitopes according to the changes of B and D antigenic regions of the HA of the influenza

vaccine strains for the past twenty years. The reassortant vaccine viruses were generated in MDCK cells. Then, their immunogenicity and efficacy in animal models was evaluated. The reassortant viruses were attenuated in mice. After immunization, rB/D influenza virus induced both humoral and cell immune responses in mice and completely protected these animals from challenge with PR8 virus. Both the virus-specific IgG and IgA titer in BAL, and the IgG in serum from mice immunized with rB/D reassortant virus were higher than the respective titers from mice immunized with other three influenza viruses. TH-1 and TH-2 type responses were found to be predominant at rB/D group. IL-4 and INF- γ responses were relatively higher in mice immunized rB/D influenza virus. This report mainly focused on obtaining high binding affinity T cell epitopes of a reassortant influenza virus, current research put emphasis on the immunicity and cross-protection of the reassortant virus.

Conclusion

The present study demonstrates that reassortant influenza virus based on HA protein changes of influenza vaccine strain could be used to produce effective cross-immunity. These results demonstrate that the rB/D influenza virus could elicit humoral immunity more efficiently. Comparably, these data on mice support the evaluation of this vaccine. It is important for a suitable match in the antigenicity of pandemic strain, according to these properties to produce vaccine in the face of an influenza pandemic.

Competing interests

The authors' declare that they have no competing interests.

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