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Immunization with baculovirus displayed H6 hemagglutinin vaccine protects mice against lethal H6 influenza virus challenge

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ABSTRACT

Low pathogenic influenza viruses of H6 hemagglutinin (HA) subtype have a high prevalence among aquatic and domestic birds and have caused outbreaks in poultry worldwide. The first human infection with wild avian influenza H6N1 virus was reported in Taiwan and these subtype viruses may continue to evolve and accumulate changes which increasing the potential risk of human-to-human transmission. To develop a vaccine against influenza viruses of the H6 subtype, we displayed the HA gene on the baculovirus surface (Bac-HA), and studied its vaccine efficacy against a lethal challenge with mouse-adapted RG-H6(Shorebird) virus carrying the H6 HA gene from A/shorebird/DE/12/2004 (H6N8) virus and 7 genes from A/Puerto Rico/8/1934 (H1N1) virus. Immunization with 256 HA units of Bac-HA via the intranasal route triggered HA-specific serum and mucosal antibodies in mice besides increased HA inhibition titers compared to mice immunized subcutaneously. Moreover, we observed an increase in cellular immune response (IL-4) and improved *in vitro* neutralization activity in the mice immunized intranasally with live Bac-HA compared to mice immunized with inactivated influenza virus (IV). Interestingly, Bac-HA intranasal immunized mice showed one fold higher neutralization titer against heterologous H6 influenza virus compared to inactivated IV immunized mice. In addition, the live Bac-HA, administered through either immunization route, as well as the adjuvanted inactivated Bac-HA, administered subcutaneously, conferred 100% protection to mice challenged with homologous mouse-adapted RG-H6(Shorebird) virus. The reduction in viral titers and extend of histopathological changes of Bac-HA immunized mice lungs further demonstrated the protective efficacy of Bac-HA. Hence, the recombinant baculovirus subunit vaccine is an alternative candidate against H6 subtypes that could be propagated and administered with minimal biosafety concerns.

1. Introduction

To date, 18 different hemagglutinin (HA) subtypes and 11 different neuraminidase (NA) subtypes have been identified in type A influenza viruses (http://www.cdc.gov/flu/about/viruses/types.htm). A limited number of HA and NA subtype influenza viruses circulate in humans and other mammalian species but all HA and NA subtype viruses are found in birds. Especially, low pathogenic avian influenza (LPAI) viruses are prevalent in aquatic and domestic birds throughout the year and are considered to be a source for novel subtypes that infect humans. For instance, the H1N1 virus that caused the 1918 catastrophic pandemic was possibly derived from an avian-like virus that had been adapted in a mammalian host (Reid et al., 2004; Taubenberger et al., 2005). The 1957 Asian H2N2 and the 1968 Hong Kong H3N2 pandemic strains were generated by reassortment between avian and human influenza viruses (Horimoto and Kawaoka, 2001). Thus, human pandemic influenza virus strains could emerge from direct transmission of avian influenza viruses or from the reassortment between an avian influenza viruses and a currently circulating human strain.

In 1997, an outbreak of A/HongKong/156/97 (H5N1) influenza virus in chickens was reported in Hong Kong, which led to 18 confirmed human cases and 6 fatalities (Claas et al., 1998; Subbarao et al., 1998; Yuen et al., 1998). Subsequent epidemiological and genetic analyses revealed that highly pathogenic A/goose/Guangdong/1/96 (H5N1), and low pathogenic A/quail/HongKong/G1/97 (H9N2) and A/teal/Hong Kong/W312/97 (H6N1) subtypes were co-circulating in much of the poultry livestock in that region (Xu et al., 1999). Since 2003, human cases of avian H5N1 infections have been reported in many parts of Asia. In addition to H5N1, avian

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viruses from other subtypes, including H7N3, H7N7, H9N2 and H10N7, have been involved in human infections (Chen et al., 2009) and recently, human infections with H7N9 and H6N1 was reported in China (Gao et al., 2013) and Taiwan (Wei et al., 2013) respectively. As it is not possible to predict with certainty which avian influenza virus subtype will cause the next pandemic, it is advisable to include low pathogenic subtypes in pandemic preparedness. Here, we selected the H6 subtype for pandemic vaccine preparation because of its high prevalence in wild birds from Europe and America (Munster et al., 2007) and in domestic poultry in China (Cheung et al., 2007). Also, the H6 viruses have caused several outbreaks in commercial poultry worldwide, thereby resulting in decreased egg production and increased mortality (Abolnik et al., 2007; Wang and Wang, 2003). Moreover, Gillim-Ross et al., 2008 suggested that H6 influenza viruses are able to bind both α-2,3- and α-2,6-sialic acid receptors based upon his observations of agglutination property of H6 viruses with red blood cells obtained from different animal species and serological examinations of US veterinarians showed significant elevation of antibody titers against H6 influenza virus (Myers et al., 2007). The recent human infection with avian influenza H6N1 virus was reported at first time from Taiwan which belonging to unique lineage in Taiwan with G228S substitution at HA gene (Wei et al., 2013).

Vaccination is a decisive measure to control the spread of the influenza viruses. Extensive research was performed for the preparation of different vaccine formulations against H1N1, H3N2, H5N1, H7N7 and H9N2 subtypes of influenza viruses (Kreijtz et al., 2009). With respect to current H6 vaccine development, only live attenuated influenza vaccine (LAIV) licensed under *Flumist* has been tested in mouse and ferret models against A/teal/HongKong/W312/97 (H6N1), A/mallard/Alberta/89/85 (H6N2) and A/duck/HongKong/182/77 (H6N9) influenza viruses (Chen et al., 2009), and clinical trials in healthy adults are underway (Talaat et al., 2011). However, LAIV has certain limitations in practical applications as it may causes side effects such as coughing, runny nose and sneezing which enables virus shedding and endangers immuno-compromised individuals in the community. The preparation of the other licensed vaccines, inactivated influenza vaccines, requires a containment facility for high pathogenic subtypes and a stable supply of specific pathogen-free eggs (Quan et al., 2010). Moreover, local or systemic allergic reactions to residual egg proteins in the vaccine components can occur in some individuals (Chung et al., 2010). Therefore, the development of a vaccine containing influenza virus membrane proteins appears to be a promising alternative and has gained special attention due to its ease of production with minimal safety concerns.

In the current study, we attempted to develop a vaccine against H6(Shorebird) reassortant virus, generated in a 7 plus 1 PR8 background carrying HA from A/shorebird/DE/12/2004 (H6N8), by displaying the HA protein on the surface of baculovirus (Bac-HA). Recombinant HA is a favorable candidate antigen for influenza vaccines, because antibodies specific to this protein elicit high neutralizing activity (Webster et al., 1982). Generally, the baculovirus expression system is considered advantageous due to higher protein yields, lower production costs, and lower level of biocontainment facilities required. However, there are some disadvantages, such as non-mammalian-like protein glycosylation and the presence of high titers of contaminating baculovirus particles and Sf9 cell debris in the expression supernatants (Krammer et al., 2010). The new trivalent seasonal influenza vaccine "*Flublok*" which contains a mixture of three recombinant hemagglutinins (rHAs) from two influenza A strains H1N1 and H3N2 and one influenza B strain that have been expressed in baculovirus was proved to be safe, immunogenic and effective in the prevention of laboratory-confirmed influenza illness (Treanor et al., 2011). For the investigation of the vaccine potential of Bac-HA, groups of mice were immunized by subcutaneous (s.c.) or intranasal (i.n.) routes and challenged with mouse-adapted reassortant H6(Shorebird) virus. We present evidence that Bac-HA immunization induces HA-specific systemic and mucosal immune responses with higher neutralizing antibody titers and protects 100% of the challenged mice. To our knowledge, this is the first report of a recombinant HA based vaccination for the LPAI H6 subtype.

2. Materials and methods

2.1. Reassortant H6 influenza viruses and pathogenicity in mice

The HA genes of A/shorebird/DE/12/2004 (H6N8) and A/duck/Hokkaido/120/2001 (H6N2) were synthesized (GenScript) based on the sequences from the NCBI influenza database. The reassortant viruses were rescued by transfecting plasmid of the HA gene (cloned into dual promoter) together with the remaining 7 gene plasmids (Hoffmann et al., 2000a) from A/Puerto Rico/8/34 (H1N1) into a coculture of 293T (ATCC, Manassas, VA, USA) and MDCK (ATCC, USA) cells. After 72 h the transfected supernatants were collected and the viral content was determined by standard HA assay as described previously (Webster et al., 1991). The reassortant viruses were confirmed by vRNA extraction followed by two step RT-PCR for the amplification of HA specific gene as described previously (Hoffmann et al., 2001). The PCR product was purified and sequenced using the BigDye Terminator v3.1 cycle sequencing kit and ABI prism 3700 DNA analyzer (Applied Biosystems, USA). The rescued viruses are termed as RG-H6(Shorebird) and RG-H6(Duck). Viruses were propagated in 10 day old specific pathogen free embryonated chicken eggs at 37 °C for 48 h. The 50% tissue culture infectious dose ($TCID_{50}$) of reassortant viruses was determined in MDCK cells (WHO, 2011) and it was calculated by Reed and Muench (1938). The RG-H6(Shorebird) virus inactivated by BEI (binary ethylenimine) (Sigma-Aldrich, St. Louis, MO, USA) as described by Sarachai et al. (2010) and inactivation was confirmed. All the above experiments were conducted in biosafety level 3 (BSL3) containment facilities, in compliance with CDCP/NIH and WHO recommendations (WHO, 2004; NIH and CDCP, 1999).

Specific pathogen free female BALB/c mice (6 weeks old) were obtained from Laboratory Animals Centre, National University of Singapore (NUS), and maintained at the animal holding unit of Temasek Lifesciences Laboratory (TLL). Mouse experiments were approved by the IACUC. A group of mice were i.n. instilled with 50 μl of RG-H6(Shorebird) influenza virus and observed for clinical sign of illness, including hunched appearance, rapid breathing and ruffled fur. The mice were sacrificed and 10% (wt/vol) lung homogenates were prepared in L-15 medium and subsequent passages were carried out as shown above. Three to four days after inoculation of the lung passage, mice presented with obvious clinical illness with hunched appearance, rapid breathing, ruffled fur and all mice showed morbidity, mortality at 8 days post challenge. The third lung passage was collected at day 7, homogenized and inoculated into chicken eggs for the virus stocks propagation and as described above. The $TCID_{50}$ and the 50% mouse lethal dose (MLD_{50}) was measured and calculated by the Reed and Muench (1938). The extraction of vRNA from lungs homogenate or allantoic fluid followed by two step RT-PCR for the amplification of each gene segments using the gene specific primers by previously described method (Hoffmann et al., 2001). Further the PCR product was cloned into pGEM-T vector as per the manufacturer's protocol (Promega, USA) and the plasmids were isolated and sequenced by BigDye Terminator v3.1 cycle sequencing kit and ABI prism 3700 DNA analyzer (Applied Biosystems, USA). The nucleotide and amino acid sequences were aligned with original sequences by ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.2. Generation of recombinant baculoviruses

For the construction of the recombinant baculovirus, HA gene of the RG-H6(Shorebird) influenza virus was cloned into modified pFASTBacHT A (Invitrogen, San Diego, CA, USA) with white spot syndrome virus immediate early promoter 1 (Gao et al., 2007) and as described by Syed Musthaq et al. (2009). The primers used for the amplification of full length ORF of HA gene from RG-H6(Shorebird) influenza virus was: Bac-HAF5′-CGCCGGTCCGATGA TTGCAATCATTGTAATAG-3′ and Bac-HAR 5′-CCGAAGCTTGGGTTAT ATACATATCCTGCACTG-3′ with *RsrII* and *HindIII* restriction enzymes. The recombinant baculoviruses were generated through site-specific transposition according to the protocol of the Bac-To-Bac system (Invitrogen, Carlsbad, CA, USA) and using insect cells, *Spodoptera frugiperda* (Sf9) (Invitrogen, USA). The recombinant baculovirus with HA gene was named as Bac-HA and without HA gene as Bac-wt. A large-scale amplification of Bac-HA was carried out and the virus particles were purified by two rounds of sucrose gradient centrifugation following standard protocols (O'Reilly et al., 1992). The recombinant baculovirus was inactivated with binary ethylenimine (BEI) as described previously (Rueda et al., 2000) and the inactivation was confirmed. The determination of the HA content present in the recombinant baculovirus was calculated by Odyssey Infrared Imager (Li-COR, Biotechnology) according to Syed Musthaq and Kwang (2011). The prokaryotic expressed, His-tag purified HA1 (PrHA1) was used as a standard and polyclonal anti-mouse PrHA1 (TLL, Singapore) acted as primary antibody for the quantitation of HA.

2.3. Immunofluorescence and Western blot assays to detect HA expression in insect cells

To detect the immunofluorescence signals, Sf9 cells were grown in 24 well plates then infected with Bac-HA or Bac-wt at a MOI of 0.5 and followed by the method described by Syed Musthaq et al., 2009. Here, 1:200 diluted 4E10 monoclonal antibody (TLL, Singapore) specific to HA of RG-H6(Shorebird) influenza virus was served as primary antibody. The fluorescence signal was detected with an inverted fluorescence microscope (Olympus, UK) and the images were captured by a digital imaging system (Nikon, USA).

For Western blot analysis, the Bac-HA infected cell supernatant was mixed with Laemmli sample buffer and resolved in 12% SDS–PAGE. The gel was transferred to a nitrocellulose membrane and Western blotting was performed as described previously (Syed Musthaq et al., 2009). The anti-mouse PrHA1 polyclonal antibody (TLL, Singapore) at a dilution of 1:5000 was used as the primary antibody and rabbit anti-mouse Ig (DakoCytomation, Denmark) at a dilution of 1:2000 was used as a secondary antibody. The protein bands were visualized by chemiluminescence kit (Amersham, UK).

2.4. Subcutaneous immunization

Six-weeks old, pathogen free, female BALB/c mice were used for vaccination experiments. Mice (5 groups/24 mice per group) were immunized via s.c. route with live Bac-HA and inactivated Bac-HA or inactivated RG-H6(Shorebird) influenza virus (IV) in the presence of Montanide ISA 201 VG adjuvant (water-in-oil-in-water emulsion; SEPPIC, France) or baculovirus wild type (Bac-wt) and PBS control at 0 and 28 days. For all baculovirus vaccine groups, mice were injected with 80 μl of 256 HA units containing 6.5 μg of HA from concentrated 1×10^8 pfu/ml recombinant baculovirus and for inactivated influenza virus groups, 80 μl of 256 HA units suspended in PBS was administered subcutaneously. Five mice from each group were anesthetized on days 28 and 42 and serum was separated and stored at −80 °C for indirect ELISA, hemagglutination inhibition (HAI) and microneutralization (MN) assays.

2.5. Intranasal immunization

Six-weeks old, pathogen free, female BALB/c mice were used for vaccination experiments. Mice (4 groups/24 mice per group) were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine and then i.n. instilled with live Bac-HA or inactivated RG-H6(Shorebird) influenza virus or Bac-wt and PBS control at 0 and 28 days. For all baculovirus vaccine groups, mice were inoculated i.n. with 80 μl of 256 HA units containing 6.5 μg of HA from the concentrated 1×10^8 pfu/ml Bac-HA and for inactivated influenza virus groups, 80 μl of 256 HA units suspended in PBS was administered. Five mice from each group were anesthetized on days 28 and 42 and serum and mucosal washes were collected and processed as described previously (Chen et al., 2001). All samples were stored at −80 °C for indirect ELISA, HAI and MN assays.

2.6. Analysis of immunogenicity of Bac-HA and RG-H6(Shorebird) vaccines in mice

The HA-specific antibody levels from serum (IgG) and mucosal (IgA) were tested separately against purified PrHA1 antigen by ELISA according to a previously described method (Bright et al., 2008). The antigen coated ELISA plates were incubated with 2-fold diluted serum samples or 1:40 diluted mucosal wash samples. The color development was then visualized by adding goat anti-mouse IgG (Sigma–Aldrich) or goat anti-mouse IgA (Bethyl Laboratories, Montgomery, TX, USA) conjugated with horseradish peroxidase to the respective wells followed by addition of 3,3′,5,5′-tetramethylbenzidine (Sigma–Aldrich). The reaction was stopped with 1 M sulfuric acid and the absorbance was measured at 450 nm using a microwell plate reader. The mean absorbance value for triplicate wells was used to express serum antibody level.

The HAI assays were performed as described previously (Webster et al., 1991). Receptor-destroying enzyme (RDE; Denka Siken Co., Japan)-treated sera were serially diluted (2-fold) in V-bottom 96-well plates. Four HA units of RG-H6(Shorebird) was incubated with serum followed by the addition of 1% chicken red blood cells (RBCs). The HAI titer was determined as the reciprocal of the last dilution that contained non agglutinated RBCs.

The MN test was performed according to previously described protocols (Suguitan et al., 2006). The heat-inactivated 42 day serum samples were mixed separately with 100$TCID_{50}$ of homologous RG-H6(Shorebird) or heterologous RG-H6(Duck) and cytopathic effect (CPE) was tracked in MDCK cells on day 4. The neutralizing titers of mouse antisera that completely prevented any CPE at reciprocal dilutions were calculated.

2.7. ELISPOT assay

At day 42, mice ($n = 3$) were euthanized and spleens were harvested and splenocytes were separated. Splenocytes (1×10^6) were added in triplicates to each well of a 96-well multiscreen HTS immuno-plate (Millipore, Billerica, MA, USA) pre-coated with purified anti-mouse interleukin-4 (IL-4) monoclonal antibody (eBiosciences, SA, CA, USA). Cells were stimulated with 1 μl/well phytohemagglutinin (PHA; Invitrogen) or 5 μl/well of 10^4 $TCID_{50}$/ml of inactivated RG-H6(Shorbird) influenza virus. Secreted IL-4 was detected by the addition of anti-IL-4 secondary antibodies coupled to biotin and avidin-HRP according to the manufacturer's protocol (eBiosciences, USA). Plates were developed by addition of 3-amino-9-ethyl-carbazole (AEC; BD Biosciences, San Jonse, CA, USA) for 10 min. Spot development was stopped by washing with distilled water. Spot forming units (SFU) were counted in an immuno-spot analyzer (Cellular Technology Limited, USA).

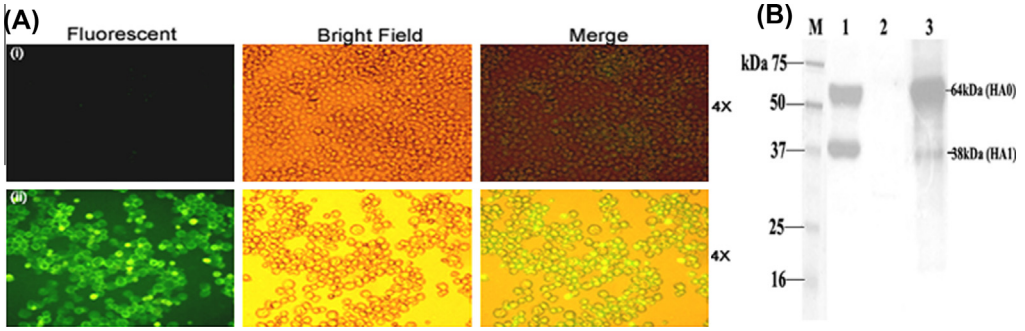

Fig. 1. Confirmation and characterization of baculovirus surface displayed HA (Bac-HA) of RG-H6(Shorebird) influenza virus by (A) immunofluorescence assay and (B) Western blot. (A) The Sf9 cells were infected with (i) wild type baculovirus (Bac-wt); (ii) Bac-HA at MOI of 0.5 and at 48 h post infection the cells were fixed and analyzed by mAb specific to HA of RG-H6(Shorebird) influenza virus to determine the antigenic confirmation of baculovirus expressed HA. (B) Western blot analysis of Bac-HA showing the cleavage of HA using anti-mouse PrHA1 (prokaryotic expression of HA1) polyclonal antibody. Lane M – broad range protein molecular weight marker; Lane 1 – Bac-HA infected supernatant; Lane 2- Bac-wt infected supernatant; Lane 3 – allantoic fluid of RG-H6(Shorebird) infected influenza virus.

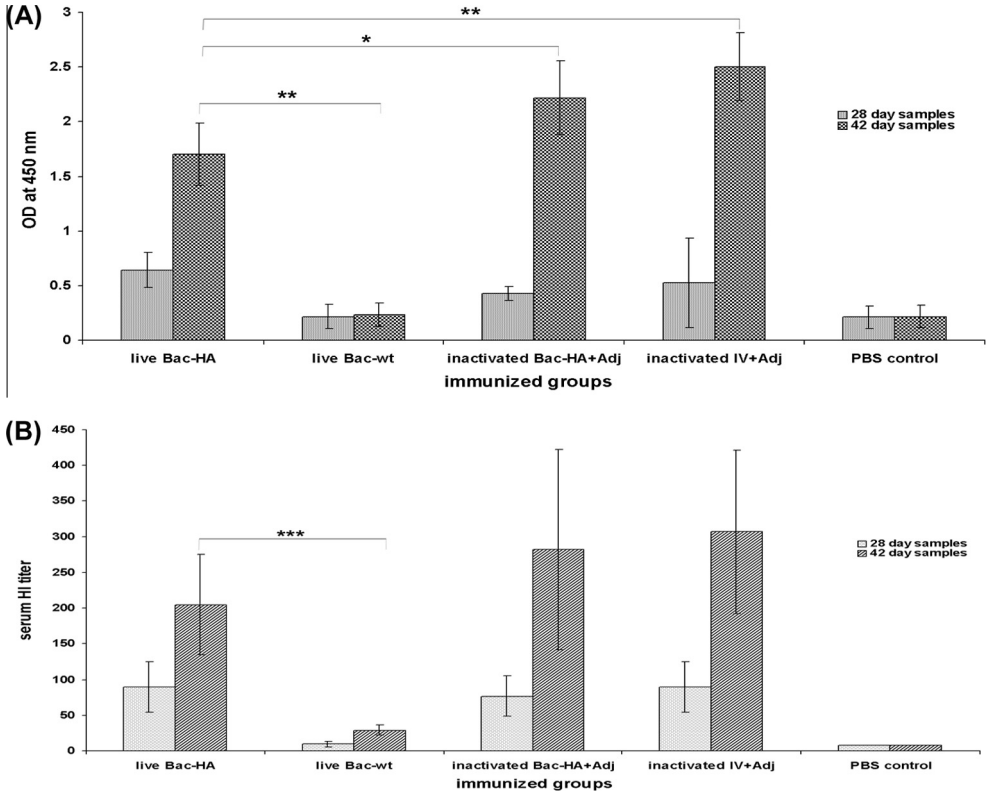

Fig. 2. Levels of systemic immune responses in the sera of mice subcutaneously immunized with different vaccine formulations. Groups of mice ($n = 10$) were immunized with live Bac-HA, adjuvanted inactivated Bac-HA, adjuvanted inactivated influenza virus (IV), live Bac-wt or PBS control on days 0 and 28. A constant dosage of 256 HA units of Bac-HA or inactivated IV suspended in PBS (80 μl/mouse) was administrated. The sera were used to determine HA-specific IgG antibody levels by indirect ELISA (A); Serum hemagglutination inhibition (HAI) titer (B). Each point represents the arithmetic mean value ($n = 5$) ± SD. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{***}P < 0.0001$. Adj – adjuvant.

2.8. Mouse challenge and Lung viral titration

Four weeks after final immunization, mice were transferred into an animal BSL3-containment facility. Fourteen mice per group of each vaccination experiment were anesthetized with a ketamine plus xylene mixture and challenged i.n. with 50 μl of 5MLD_{50} of homologous mouse-adapted RG-H6(Shorebird) virus. Mice were observed daily for mortality and body weight was monitored on alternate days. Monitoring continued until all control mice died or until day 18 after challenge. The viral titers were measured from the mice lungs harvested on day 3, 6 and 9 after challenge and as described (WHO, 2011). Briefly, each mice lung was used to prepare as clarified homogenates in 1 ml PBS and inoculated into MDCK cells. Four days after incubation, the infected cells were observed under microscope and $TCID_{50}$ was calculated by Reed and Muench (1938).

2.9. Histopathological analysis

The mice were sacrificed 8 days after challenge, and lungs were collected in 10% (wt/vol) buffered formalin, embedded and sectioned. The sections were deparaffinized using Hist-choice (Amersco, USA) and rehydrated in sequentially graduated ethanol baths. The sections were stained with hematoxylin & eosin and sections were mounted using glycerol. The slides were observed under a microscope (Olympus, UK) and the images were captured by digital imaging system (Nikon, USA).

2.10. Statistical analysis

The data were expressed as arithmetic mean ± standard deviation. The unpaired two-tailed Student's t test was performed to determine the level of significance in the difference between means of two groups. Statistical significance was expressed as $P < 0.05$.

3. Results

3.1. Pathogenicity of reassortant RG-H6(Shorebird) influenza virus in mice

Based on the year of virus isolation and avian species, two H6 influenza viruses (H6N8 and H6N2) were chosen and rescued by reverse genetics method. However, only RG-H6(Shorebird) virus was used for mouse pathogenicity study and subsequent experiments, whereas RG-H6(Duck) virus utilized for the analysis of neutralizing antibody titers against heterologous virus. For mice pathogenicity study, the mice were inoculated with a 50 μl of $10^{4.3}$ $TCID_{50}$/ml of RG-H6(Shorebird) virus and at day 6, virus inoculated mice showed mild clinical signs of illness with a 5% weight loss compared with PBS inoculated mice, but no mortality was recorded. At day 7 post inoculation of passage one, 10% (w/v) of mice lung homogenate was prepared and 50 μl of $10^{4.6}$ $TCID_{50}$/ml viral titer was inoculated into mice for a second passage and the clinical observation was continued. At day 8 of second passage RG-H6(Shorebird) infected mice presented with significant weight loss of up to 12% of their original body weight, with moderate illness starting at day 4, whereas PBS inoculated mice showed no clinical sign of illness. At day 7 post inoculation of passage two, 10% (w/v) mice lung homogenate was prepared and 50 μl of $10^{5.4}$ $TCID_{50}$/ml viral titer was inoculated for a third passage and continued the clinical observation. From the third passage onward, the RG-H6(Shorebird) infected mice showed the obvious clinical illness such as hunched appearance,

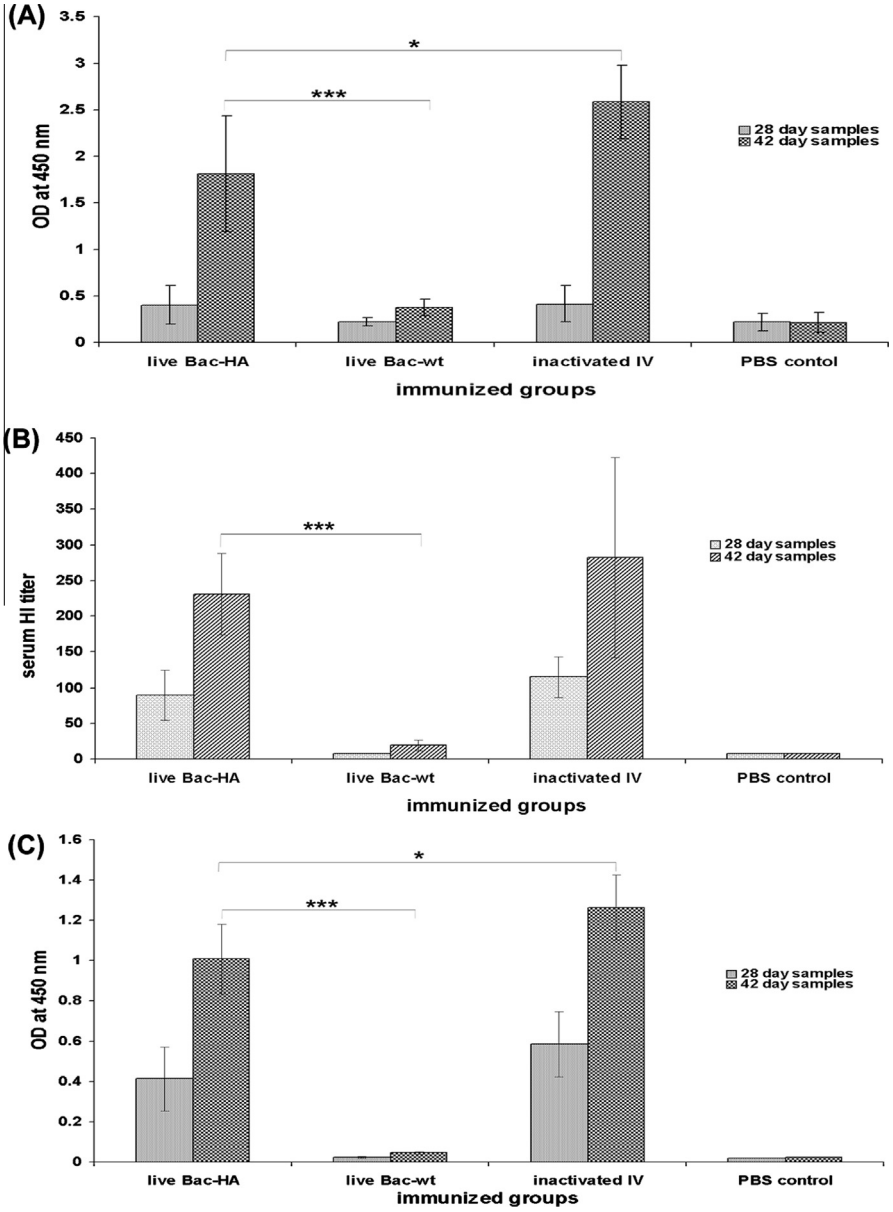

Fig. 3. Levels of systemic and mucosal immune responses in the sera of mice intranasally immunized with different vaccine formulations. Groups of mice ($n = 10$) were i.n. immunized with live Bac-HA, inactivated IV, live Bac-wt or PBS control on days 0 and 28. A constant dosage of 256 HA units of Bac-HA or inactivated IV suspended in PBS (80 μl/mouse) was administrated. The sera were used to determine HA-specific IgG antibody level by indirect ELISA (A); Serum hemagglutination inhibition (HAI) titer (B); HA-specific IgA levels by indirect ELISA (C). Each point represents the arithmetic mean value ($n = 5$) ± SD. $^{*}P < 0.05$; $^{***}P < 0.0001$.

rapid breathing and ruffled fur with significant weight loss starting at day 3. At day 8 post challenge, all mice lost up to ⩾20% of their body weight and succumbed. The viral titer of third lung passaged mouse-adapted RG-H6(Shorebird) was determined as 10^6 $TCID_{50}$/ml. Further, the third passaged mice lung supernatant was propagated in the allantoic cavity of eggs and $10^{6.1}$ $TCID_{50}$/ml viral titer was determined, which is representing a 1.8 $\log_{10}TCID_{50}$/ml increase compared to the original RG-H6(Shorebird) and maintained as stock virus for the further studies. Sequencing of mouse-adapted RG-H6(Shorebird) influenza virus did not showed any mutations in HA gene with H6N8 influenza virus. However, among the other seven genes of mouse-adapted RG-H6(Shorebird) virus, the mutations were identified only at PB1 gene (A53G, S216G, L300F, H473L and S517I) and PB2 gene (Y360F, L619F), when compared with A/Puerto Rico/8/34 virus. Next, the MLD_{50} of RG-H6(Shorebird) was determined and the challenge dosage was calculated ($5MLD_{50} = 10^{2.5}$ $TCID_{50}$/ml).

3.2. Generation of a recombinant baculovirus expressing HA and the confirmation of HA function and antigenicity

The expression of HA from Bac-HA was analyzed by immunofluorescence method and the results indicated that the baculovirus encoded HA was expressed on Sf9 cells infected with Bac-HA. In contrast, no fluorescence signal was observed in Bac-wt infected insect cells (Fig. 1A). To examine the molecular mass of HA incorporated into budded recombinant baculoviruses, we collected the supernatant of baculovirus infected cells and assessed it in a Western blot, which detected two bands with molecular masses of 64 and 38 kDa corresponding to HA0 and HA1 respectively (Fig. 1B). These signals were not detected in the Bac-wt derived supernatants. Further, to determine whether the HA expressed by baculovirus system was properly translocated to the baculovirus surface, Bac-HA and Bac-wt infected cell culture supernatants were incubated with RBCs to monitor agglutination activity. The baculovirus expressed HA was absorbed by RBCs, and a clear agglutination activity of up to 128 HA units was observed. In contrast, no RBCs absorption was observed for the supernatant from uninfected cells or from insect cells infected with Bac-wt. This result shows that surface displayed HA has receptor binding activity, which suggests that the HA protein is properly folded.

3.3. Immunogenicity of Bac-HA and RG-H6(Shorebird) vaccines in mice

To monitor systemic immunity, the serum HA-specific IgG antibody levels were measured in all immunized groups and it highly elevated at day 42 compared to live Bac-wt or PBS control that shows an efficient booster effect (Figs. 2 and 3A, $P < 0.001$). The slightly higher serum HA-specific IgG levels observed for s.c. injected adjuvanted inactivated Bac-HA and IV compared with live Bac-HA can be explained by the synergistic effect of Montanide ISA 201 VG adjuvant (Fig. 2A, $P < 0.03$ and $P < 0.002$, respectively). While i.n. immunizations of live Bac-HA resulted in an increased serum HA-specific IgG levels at day 42 compared to s.c. immunization, this level was still lower than that of the inactivated IV group (Fig. 3A, $P < 0.04$).

Next, the HI tiers was assayed in all mice groups and had significantly higher at day 42 ($P < 0.0001$) compared with Bac-wt and PBS control groups, but no significant difference was observed between them (Figs. 2 and 3B). After s.c. immunizations, the live Bac-HA and adjuvanted inactivated form of either Bac-HA or IV elicited mean HAI titers of 204.8, 281.6 and 307.2, respectively on day 42 (Fig. 2B). The HAI titer of live-Bac i.n. immunized mice was marginally elevated compared to s.c. immunization which corresponds to the increase in serum HA-specific IgG levels of live Bac-HA i.n. immunized mice (Fig. 3B). The mean HI value of live Bac-HA (230.4) was comparable to inactivated IV (281.6) (Fig. 3B). In conclusion, slightly lower serum HA-specific IgG levels in the live Bac-HA immunized animals translated into a similar HI activity of the functional antibodies.

Levels of HA-specific mucosal IgA were tested in the i.n. immunized mice with live Bac-HA and inactivated IV and it elevated at day 42 followed by a booster effect on day 28 (Fig. 3C, $P < 0.0001$). The mucosal antibody levels of the live Bac-HA group were slightly lower than those of the inactivated IV group (Fig. 3C, $P < 0.04$). The HA-specific serum IgG and mucosal IgA levels of Bac-wt and PBS control mice were negligible at both time points, regardless of the immunization route.

Finally, the determined neutralizing antibody titers of Bac-wt or PBS control mice were negligible regardless of the immunization route or virus strain (Table 1). Neutralization titers against homologous RG-H6(Shorebird) only had less than 1-fold difference between the vaccinated groups: Sera from mice immunized s.c. with adjuvanted inactivated Bac-HA or adjuvanted inactivated IV had a neutralization titer of 1173 and 1280, respectively compared to 747 for live Bac-HA, as expected from the lower levels of serum HA-specific IgG in this group. Interestingly, this result was reversed following i.n. immunization where live Bac-HA elicited the higher neutralization titer (1706) than inactivated IV (853) ($P < 0.05$). An ever bigger advantage of i.n. live Bac-HA vaccination was observed in the neutralization titer against heterologous RG-H6(Duck) virus where a titer of 1174 was observed for live Bac-HA in contrast to inactivated IV at 587 corresponding to a one fold increase (Table 1, $P < 0.001$). This could be due to fact that both RG-H6(Shorebird) and RG-H6(Duck) viruses shared 98.7% amino acid identities in the HA protein, which is a difference in seven amino acids were occurred in the HA of RG-H6(Shorebird) virus when compare with RG-H6(Duck) influenza virus. Exclusively three amino acids were existed in the antigenic site (H3/H1 numbering) of HA of H6N8 influenza virus. Amino acid (Ile) at position 127 corresponds to antigenic site A of H3 numbering and amino acids (Gln, Ser) at positions 149, 260 corresponds to antigenic sites Ca2[b] and Cb of H1 numbering respectively. Next, the H6 HA phylogenetic tree revealed that A/shorebird/DE/12/2004 (H6N8) and A/duck/Hokkaido/120/2001 (H6N2) viruses are clustered into major H6 influenza virus subtypes (Fig. 4).

Table 1
Virus neutralizing antibody titers of subcutaneous or intranasal immunized mice sera against homo- and heterologous RG viruses.

Immunization route/groups	Serum neutralizing antibody titers	
	RG-H6(Shorebird)	RG-H6(Duck)
A. Subcutaneous immunization		
Live Bac-HA	747	533
Adjuvanted inactivated Bac-HA	1173[a,*]	960[a,**]
Adjuvanted inactivated IV	1280[a,**]	1173[a,**]
Live Bac-wt	12	10
PBS control	<	<
B. Intranasal immunization		
Live Bac-HA	1706[b,*]	1174[b,**]
Inactivated IV	853	587
Live Bac-wt	13	10
PBS control	<	<

$100TCID_{50}$ of each RG-H6(N8) or RG-H6(N2) influenza viruses were used in this study; Sera obtained from three mice/group on 42 day; values are represented as geometric mean titers; <, neutralizing antibody titer below the detection limit 1:10.
[*] $P < 0.05$.
[**] $P < 0.01$.
[a] When compared with live Bac-HA in s.c. immunization.
[b] When compared with inactivated IV in i.n. immunization.

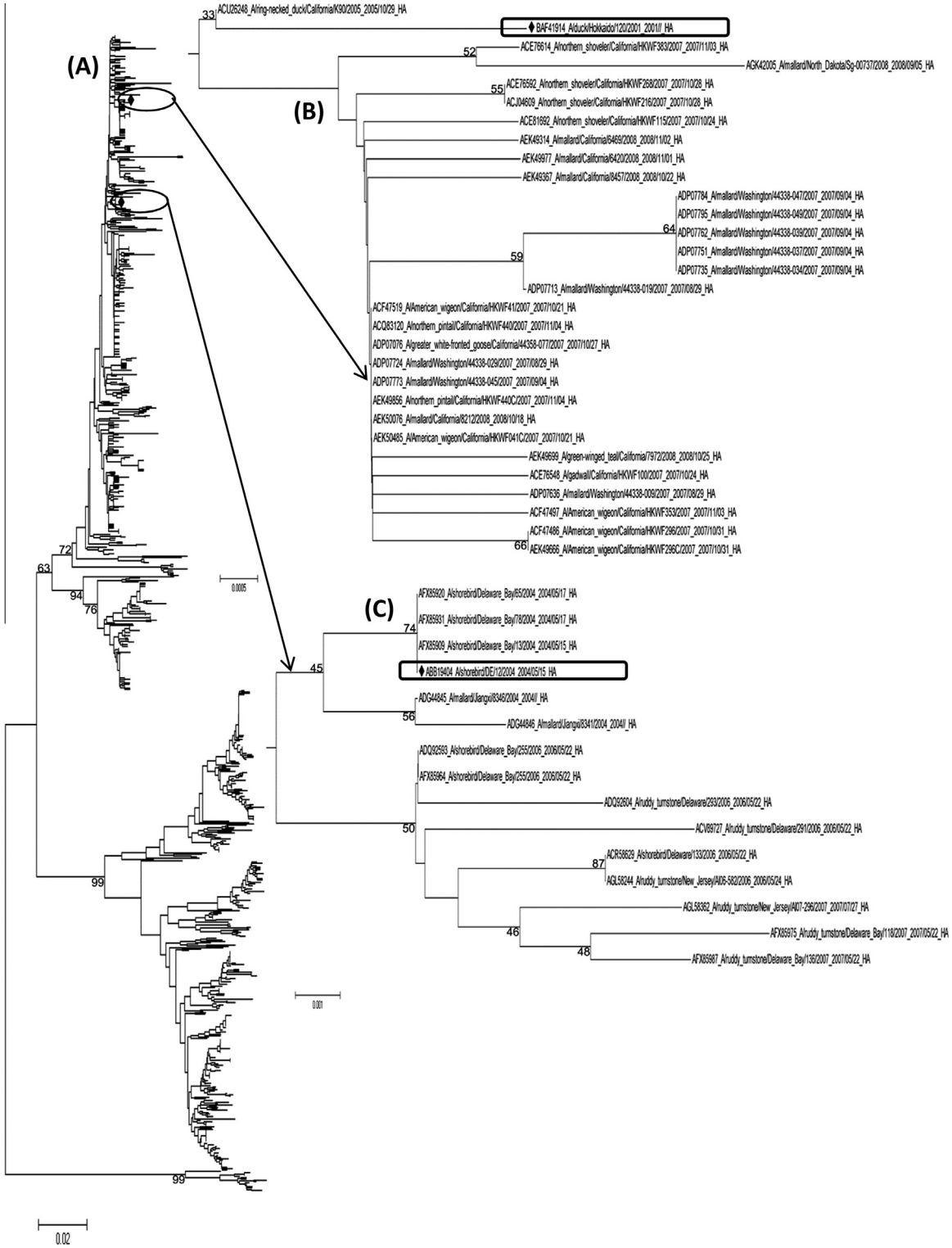

Fig. 4. Phylogenetic tree of all full length H6 HA amino acid sequences of avian influenza viruses was inferred using Neighbor-Joining method. (A)-Aggregated tree was built for a dataset containing 904 full length HA protein for Influenza A H6 viruses extracted from avian hosts during the period 2000–2014; (B and C)-Two full-resolution trees are expanded from aggregated tree for the indication of A/duck/Hokkaido/120/2001 (H6N2) and A/shorebird/DE/12/2004 (H6N8) subtype viruses (in rectangular box) respectively. The trees were constructed using MEGA5 and the bootstrap values were generated using Poisson correction method with the implemented 1000 replicates.

3.4. Cellular immune response to Bac-HA and RG-H6(Shorebird) immunization in mice

The cellular immune response, IL-4 cytokine expression level was highly elevated in all the vaccinated groups compared to the Bac-wt or PBS controls (Fig. 5A and B, $P < 0.0001$). For the s.c. immunization, the IL-4 level of adjuvanted inactivated Bac-HA mice (SFU < 236) was slightly higher than that of adjuvanted inactivated IV counterparts (SFU < 201, $P < 0.02$) and higher than that of live Bac-HA (SFU < 170, $P < 0.01$) (Fig. 5A). Similar to the observations of serum HA-specific IgG and HI levels, the live Bac-HA immunized mice had a significant increase in IL-4 responses after

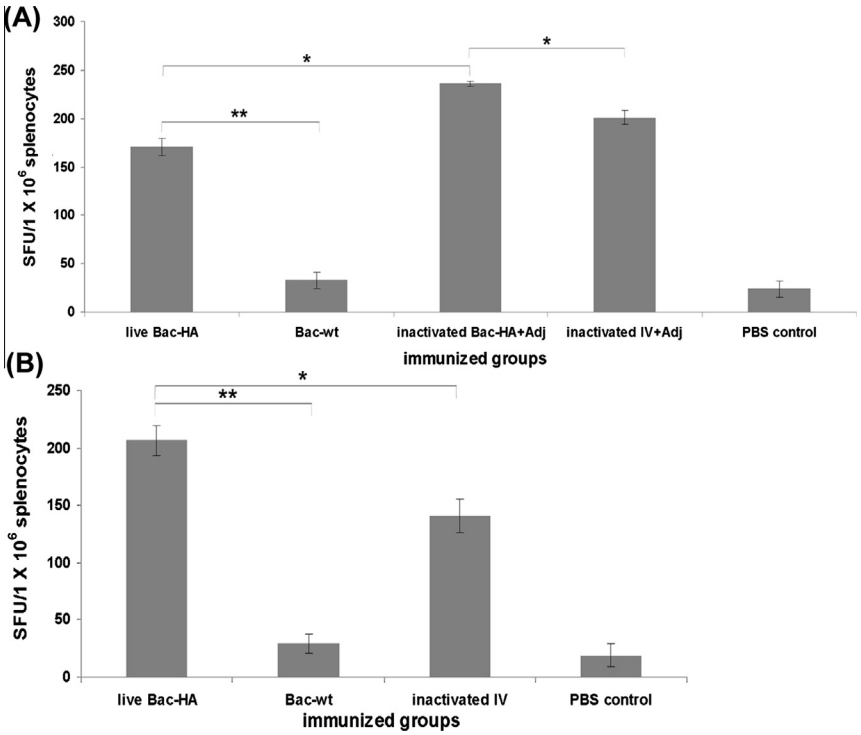

Fig. 5. ELISPOT results of IL-4 response of (A) subcutaneously (B) intranasally immunized mice. Splenocytes were stimulated with inactivated RG-H6(Shorebird) influenza virus. Spleen cells were isolated from the various mice groups (n = 3/each group) of either s.c. or i.n. immunization described in the legend of Figs. 2 and 3 on day 42 and were pooled to determine the IL-4 response. The statistical significant of the difference between groups was calculated by student's t test. $^{*}P < 0.05$; $^{**}P < 0.001$. SFU – spot-forming units; Adj – adjuvant.

i.n. administration (SFU < 206, $P < 0.05$) compared to s.c. injection (Fig. 5B). The i.n administration of live Bac-HA also resulted in significantly higher IL-4 levels than in the inactivated IV group (SFU < 140, $P < 0.04$, Fig. 5B) and these levels were comparable to the s.c. injected adjuvanted inactivated IV group.

3.5. Protective efficacy of Bac-HA vaccine against lethal challenge with RG-H6(Shorebird) influenza virus

Groups of mice challenged after s.c. immunization with live Bac-HA, inactivated adjuvanted Bac-HA and inactivated adjuvanted IV all conferred 100% protection throughout the 16 day observation period (Fig. 6A). Similarly, i.n. immunization with live Bac-HA and inactivated IV resulted in 100% survival (Fig. 7A) while mice immunized with Bac-wt or PBS by both routes succumbed to infection starting at 2–3 days post challenge with obvious clinical signs of illness including hunched appearance, rapid breathing and ruffled fur. Their body weights declined rapidly up $\geqslant$20%, and all the mice died at 8 days post challenge. The weight loss in survived mice immunized both s.c. and i.n., however, was only around 5% for all vaccine constructs. S.c. immunized mice started to regain their weight at day 5–6. (Fig. 6B). The body weights of the i.n. immunized live Bac-HA or inactivated IV groups reached their lowest of 5.08% and 4.73% loss, respectively, at 4 days post challenge with a rapid return to normal from day 5 onwards (Fig. 7B).

The analyzed lung viral titers of PBS control or Bac-wt groups of either immunization route were elevated between $10^{5.6}$ and $10^{5.8}$ $TCID_{50}$/g on 6 dpi from $10^{4.1}$ to $10^{4.3}$ $TCID_{50}$/g on 3 dpi, which demonstrated the efficient replication of RG-H6(Shorebird) virus in mice lungs (Fig. 8A and B). The lung viral titer of i.n. immunized live Bac-HA dropped to $10^{2.3}$ $TCID_{50}$/g on 9 dpi compared to 3 dpi and also it maintained comparably less viral titer with inactivated IV group (Fig. 8A). Whereas, among the s.c. immunized groups adjuvanted inactivated Bac-HA mice viral titer was dropped to $10^{2.3}$ $TCID_{50}$/g which is less with live Bac-HA groups and comparable with adjuvanted inactivated IV on 9 dpi (Fig. 8B). The pathology of Bac-wt and PBS control mice lung tissues showed infection initiating with cellular debris in the bronchiolar lumen and severe alveolar collapse throughout the lungs, which resulted in deformation of the whole lung (Fig. 9A and B). In contrast, the lungs of protected mice from the live Bac-HA (Fig. 9C and F), adjuvanted inactivated baculovirus (Fig. 9D) or inactivated IV (Fig. 9E) of either immunization showed minimal bronchitis and a reduction or absence of lesions in the examined lung tissue.

4. Discussion

A pandemic influenza virus could be generated by reassortment between an avian influenza virus and a currently circulating human strain. H6 subtypes are prevalent in domestic poultry and wild birds throughout the year and are able to infect both avian and mammalian species which enhances the possibility of the H6 subtypes contributing to reassortant strains. Therefore, pandemic preparedness for LPAI, especially for the H6 subtype, is an essential step in controlling the spread of influenza virus outbreaks. In the present study, low pathogenicity of the first passage of RG-H6(Shorebird) virus that we observed in mouse is in accordance with the findings of Hoffmann et al. (2000b), who reported initial low pathogenicity of A/teal/HongKong/W312/97 (H6N1) virus in mouse due to the absence of a highly cleavable HA molecule. They further suggested that the later rapid increase in pathogenicity was due to other gene segments also play important role or the mice that were inoculated with high dose (8.5 EID_{50}) of virus (Hoffmann et al., 2000b). Further Gillim-Ross et al. (2008) observed a difference in clinical illness as well as mortality percentage in mice that were inoculated with various $TCID_{50}$ dilutions

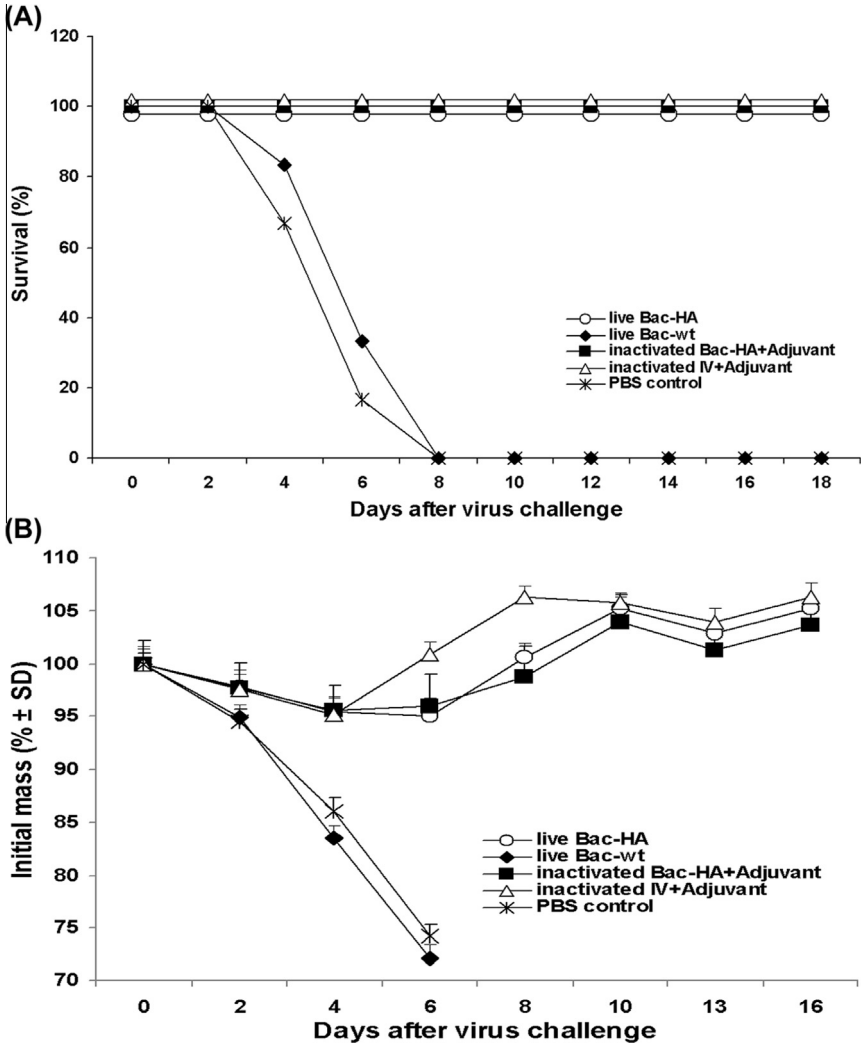

Fig. 6. Protection of subcutaneously immunized mice from lethal RG-H6(Shorebird) influenza virus challenge. The immunized mice ($n = 6$/groups) on days 0 and 28 as indicated in the legend of Fig. 2 were challenged i.n. with $5MLD_{50}$ of mouse-adapted RG-H6(Shorebird) virus on day 56. Mice were monitored for survival through a 18 days observation period, and the results were expressed as percent survival (A); the weight loss of the mice groups was also monitored throughout a 16 days observation period and the results were expressed in percent body weight (B) compared to the beginning of the trial.

of two highly replicative nature H6N1 influenza viruses. Nevertheless, in the present mice pathogenicity study, 100% mortality was observed in RG-H6(Shorebird) inoculated mice at third mice lung passage. Hence, it is presumed that together with the increased viral titer, the mutations at PB1 and PB2 genes might be responsible for the mouse pathogenicity by RG-H6(Shorebird), however, further study needed to confirm the molecular mechanism.

In the current study, we displayed the HA protein of RG-H6(Shorebird) influenza virus on the baculovirus surface and assessed its vaccine efficacy in a mouse model by s.c. or i.n. immunizations. Many viral envelope proteins are effectively displayed on the baculovirus surface and applied as vaccine candidates against viral infections (Prabakaran et al., 2008; Li et al., 2009a,b; Yoshida et al., 2010). Also several studies have indicated high level of anti-HA antibodies in mouse sera obtained from baculovirus surface-displayed HA vaccination against H1N1 and H5N1 influenza viruses (Prabakaran et al., 2010, 2011; Yang et al., 2007). Similarly, we observed high HA-specific serum antibodies and HAI titers after the boost in all the immunized groups independent of the administration route. Interestingly, live Bac-HA elicited better immune responses when given i.n. instead of s.c. which might be due to the fact that baculovirus DNA itself can act as an adjuvant by stimulating a robust immune response in the respiratory tract (Hervas-Stubbs et al., 2007). Additionally, HA-specific IgA levels were clearly elevated in the i.n. live Bac-HA and inactivated IV immunized mice confirming that vaccination via the mucosal route stimulates both systemic and mucosal immune responses (Horimoto and Kawaoka, 2001) while a mucosal immune response is normally undetectable in s.c. vaccinated mice (Kaul and Ogra, 1998; McGhee et al., 1992). A putative vaccine has to be able to induce virus neutralizing antibodies, ideally against both homologous and heterologous virus strains. The neutralizing capacity of the s.c. immunized live Bac-HA sera was one fold lower than that of adjuvanted inactivated IV corresponding to a lower HA-specific IgG level. This was contrary to the results of the i.n. immunization where the neutralization titer of live Bac-HA sera against homologous RG-H6(Shorebird) influenza virus was enhanced one fold compared with the neutralizing activity of the inactivated IV sera. Especially, the one fold increase in cross-neutralization of heterologous RG-H6(Duck) by live Bac-HA i.n. vaccination compared with inactivated IV marks this vaccine as an alternative for heterologous H6 influenza virus protection. Moreover, the H6 HA phylogenetic tree and examined antigenic sites of RG-H6(Shorebird) virus supports the cross-neutralization activity of RG-H6(Shorebird) serum

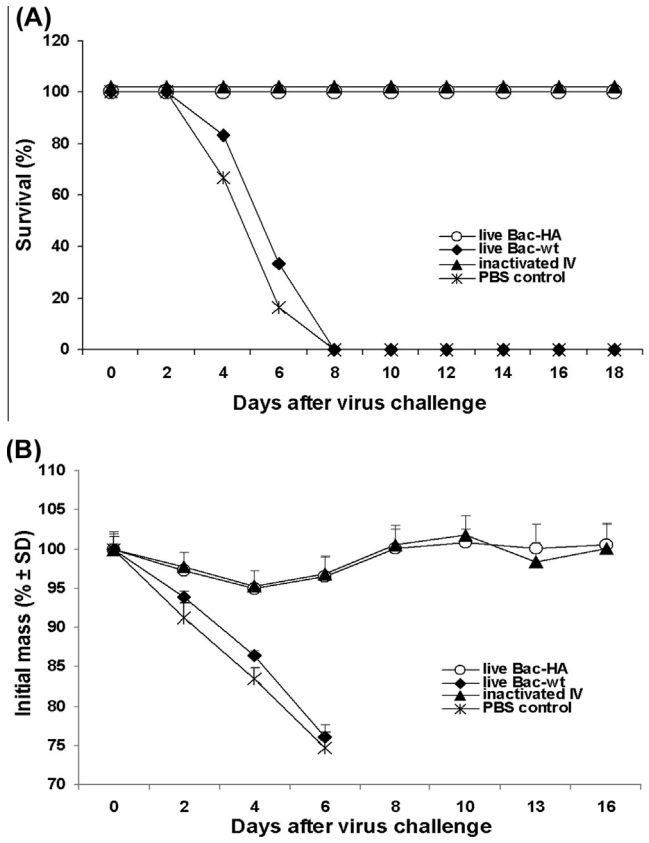

Fig. 7. Protection of intranasally immunized mice from lethal RG-H6(Shorebird) influenza virus challenge. The immunized mice (n = 6/groups) on days 0 and 28 as indicated in legend of Fig. 3 were challenged i.n. with 5MLD$_{50}$ of mouse-adapted RG-H6(Shorebird) virus on day 56. Mice were monitored for survival through a 18 days observation period, and the results were expressed as percent survival (A); the weight loss of the mice groups was also monitored throughout a 16 days observation period and the results were expressed in percent body weight (B) compared to the beginning of the trial.

against RG-H6(Duck) virus and it might be extends to other subtypes in the cluster. However further studies are needed to authenticate the reported or other antigenic amino acids involved in the neutralizing activity of RG-H6(Shorebird) virus.

Cellular immunity is also induced by inactivated influenza immunization and is important in anti-influenza virus activity (Bender et al., 1995). Along with the ability of recombinant baculovirus to efficiently transduce mouse cells (Tani et al., 2003), the i.n. immunizations induces higher amounts of Th1-type and Th2-type cytokine responses than s.c. immunization (Chen et al., 2004; Giri et al., 2005). So, in the present study we observed increased levels of Th2-type (IL-4) cytokine production in the live Bac-HA i.n. immunized mice compared with s.c. immunization. Also the IL-4 levels were significantly elevated in i.n. immunized live Bac-HA compared to inactivated IV immunized mice. It has been shown that inoculation with recombinant baculovirus can stimulate the secretion of inflammatory cytokines such as TNF-α, IL-1 α, IL-6, IL-12 and IL-1 β in mice (Abe et al., 2003). The IL-4 level of adjuvanted inactivated Bac-HA is marginally higher than that of adjuvanted inactivated IV which might indicate that together with recombinant baculovirus the Montanide-adjuvant stimulates humoral and cellular immune responses that significantly improve IgG2 production and increase Th1-type responses (SEPPIC, France). However, the effects of i.n. immunization of inactivated Bac-HA in the presence of a suitable adjuvant remain to be elucidated.

The protective efficacy of s.c. immunization with live Bac-HA or adjuvanted inactivated Bac-HA or IV and of i.n. immunization with both live Bac-HA and inactivated IV was 100% with minimal weight loss. To further authenticate these findings, we determined viral titers and examined histological changes in the lungs of mice from different experimental groups. Despite viral titers were slightly elevated in the baculovirus vaccinated group on 3 dpi, low viral titer of below $10^{2.4}$ was determined at 9 dpi, indicates mice were successfully recover from viral infection that observed by gaining of body weight without any clinical signs of illness. Whereas, higher viral titers were recorded on Bac-wt and PBS control groups on 6 dpi, and later all mice were succumbed to death at 8 dpi. The findings of lung viral titer were correlated with previous baculovirus vaccinations against H5N1 influenza virus (Tang et al., 2010). The histopathology of survivors presented with minimal or no lesions, whereas in control mice (Bac-wt or PBS) cell debris accumulated of in the bronchiolar lumen, as well as the enlargement of the alveolar ducts followed by severe alveolar collapse in the whole lungs (Fukushi et al., 2011). This observation was consistent with baculovirus vaccination against H1N1 (Prabakaran et al., 2011). The 100% survival rate achieved in the present vaccination successfully demonstrated that baculovirus surface displayed HA of RG-H6(Shorebird) is an efficient vaccine candidate for LPAI. As our *in vitro* MN assay showed strong cross-neutralization of i.n. live Bac-HA to heterologous H6 strains, it would be informative to also carry out a heterologous challenge experiment in the future.

The results obtained in this study mirror previous reports of baculovirus immunizations against influenza virus subtypes H5N1, H1N1 (Crawford et al., 1999; Prabakaran et al., 2008, 2010, 2011) and for LPAI non-H6 subtypes (Lin et al., 2011; Crawford et al., 1999). Concerning H6 vaccination, LAIV (*FluMist*) vaccines for influenza viruses of H6N1, H6N2 and H6N9 subtypes

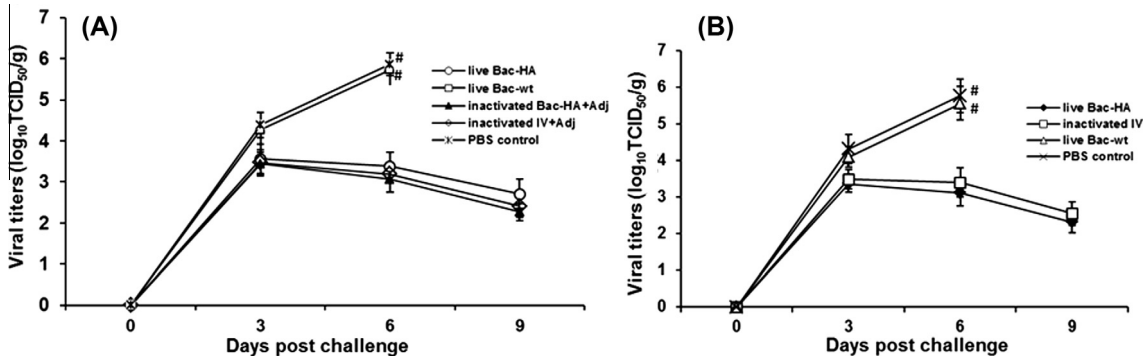

Fig. 8. Viral titer measurement in lungs of mice challenged with 5MLD$_{50}$ of RG-H6(Shorebird) virus after subcutaneous (A) and intranasal (B) immunizations. The lung tissues of mice obtained from the challenged mice groups at day 3, 6 and 9 which are immunized previously by s.c. or i.n. routes as described in the legend of Fig. 2 or Fig. 3, respectively. The results of viral titers in lungs were expressed in terms of mean value of log$_{10}$TCID50/g. # Represents no survival of any mice in the groups.

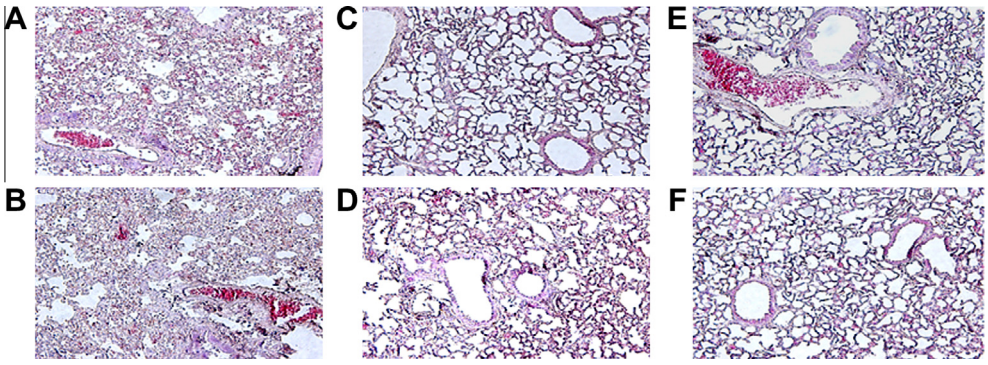

Fig. 9. Histopathological changes in lung sections of mice challenged with $5MLD_{50}$ of RG-H6(Shorebird) virus at day 8 after subcutaneous or intranasal immunizations. The lung tissues of mice obtained from challenged mice groups which are immunized previously by s.c or i.n. routes previously as described in the legends of Fig. 2 or Fig. 3, respectively. The control groups of both immunization routes had severe alveolar collapse throughout the lungs tissue: live Bac-wt (A) and PBS (B). On the other hand, s.c. immunization with live Bac-HA (C), adjuvanted inactivated Bac-HA (D), and adjuvanted inactivated IV (E) or i.n. immunization with live-Bac-HA (F) resulted in minimal or no lesions in the examined lung sections.

have been prepared and were evaluated in mouse and ferret models where only the H6N1 vaccine was shown to fully protect against homologous and heterologous (H6N2, H6N9) wild-type virus challenge (Chen et al., 2009). However, the HA-specific serum and mucosal antibodies were not studied for the LAIV vaccine. It is presumed that H6 influenza subtype infections initiate as a result of replication in the upper respiratory tract, thus causing clinical illness in experimental infections (Beare and Webster, 1991). As indicated in our mouse vaccine study, i.n. immunization with live Bac-HA stimulated the production of HA-specific serum and mucosal antibodies as well as increased survival rate. Therefore, we hypothesize that in i.n. immunization, the secretion of HA-specific mucosal antibodies preventing upper respiratory tract infections and HA-specific serum antibodies preventing viral pneumonia, that may lead in the circumventing a pathogenic RG-H6(Shorebird) influenza virus infection in mice.

In summary, we demonstrated that a lung passaged RG-H6(Shorebird) influenza virus strain causes 100% mortality in mice within 8 days after infection. The live Bac-HA administered by both i.n. and s.c. immunization routes or adjuvanted inactivated Bac-HA administrated s.c. protected mice completely against a lethal RG-H6(Shorebird) challenge, which was equal to adjuvanted inactivated IV counterpart. However, the neutralizing titer and IL-4 responses in the i.n. immunized live Bac-HA were improved compared to those of the s.c. immunized adjuvanted inactivated IV, suggesting that recombinant baculovirus displayed HA is a suitable vaccine candidate for LPAI infections. The good safety profile of baculovirus, its easy propagation without biocontainment facilities and its ability to induce systemic, mucosal and cellular immune responses (Treanor et al., 2007) make recombinant baculovirus an attractive vaccine strategy. Because only live attenuated influenza vaccination trials have been evaluated for the H6 subtype, this recombinant baculovirus vaccination could be considered as an alternative vaccine platform for H6 influenza viruses.

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