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Protective efficacy of an H1N1 cold-adapted live vaccine against the 2009 pandemic H1N1, seasonal H1N1, and H5N1 influenza viruses in mice

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ABSTRACT

Vaccination is a key strategy for preventing influenza virus infections. Here, we generated a reassortant virus (SC/AA*ca*) containing the hemagglutinin and neuraminidase genes from a 2009 pandemic influenza virus A/Sichuan/1/2009 (H1N1) (SC/09) and six internal genes from the cold-adapted virus A/Ann Arbor/6/60 (H2N2) (AA*ca*). The SC/AA*ca* reassortant induced a sound humoral immune response and complete protection against homologous SC/09 virus challenge in mice after intranasal administration of an at least 10^6 50% egg infectious dose (EID_{50}) of SC/AA*ca*. SC/AA*ca* inoculation also induced significant CD4+ and CD8+ T cell responses and provided solid protection against heterologous H1N1 and H5N1 virus challenge. Our results suggest that this 2009 H1N1 live vaccine will provide protection against both 2009 pandemic and seasonal H1N1 virus infection and might reduce the severity of H5N1 virus infection in humans. The induction of cross-reactive virus-specific T cell responses may be an effective approach to develop universal influenza vaccines.

1. Introduction

Influenza A viruses are divided into subtypes on the basis of the antigenicity of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA); 15 HA and 9 NA subtype viruses have been isolated from birds, but only H1N1, H2N2, and H3N2 subtype viruses have circulated widely and caused epidemics in humans in the last century. Beginning in late 2003, outbreaks of H5N1 influenza A virus infection occurred among poultry and wild birds in numerous Asian countries, with subsequent reports in Europe and Africa (Office International des Epizooties [OIE]; http://www.oie.int). Despite substantial infection control efforts, H5N1 viruses have continued to evolve and spread, producing human infections in 15 countries, with 329 of the 562 confirmed cases proving fatal (World Health Organization [WHO]; http://www.who.int). These viruses thus pose a significant pandemic potential to public health. In April 2009, an antigenically distinct swine-origin H1N1 influenza A virus was detected in humans (CDC, 2009). This novel H1N1 virus, referred to as the pandemic 2009 H1N1 virus (2009 H1N1), spread efficiently around the world, leading the WHO to declare a global pandemic on June 11, 2009 (World Health Organization [WHO]; http://www.who.int). Although infection with the 2009 H1N1 virus causes a mild, self-limiting respiratory illness in most people, the young and those with certain underlying conditions, including asthma, diabetes, heart/lung problems, morbid obesity, and pregnancy, are at greater risk of severe disease progression (Jain et al., 2009).

Vaccination is an important strategy to protect humans against influenza viruses. Whole virus inactivated vaccines, split vaccines, subunit vaccines, virus-like particle vaccines, DNA vaccines and live attenuated vaccines have all been developed and tested in animal models or humans (Belongia et al., 2009; Girard et al., 2010a; Pearce et al., 2011; Verity et al., 2011; Wen et al., 2009; Yang et al., 2011). Both inactivated and live attenuated vaccines have been used in humans against the H1N1 and H3N2 subtype influenza viruses. Compared with inactivated vaccines, live attenuated vaccines generally induce broadly cross-reactive protection against different strains within the same subtype, and partial protection against other virus subtypes has been observed in mice (Suguitan et al., 2006).

The cold-adapted (*ca*) influenza virus A/Ann Arbor/6/60 (AA) (H2N2) has been developed as a live attenuated vaccine seed virus that exhibits cold-adaptation, temperature-sensitivity (*ts*), and attenuation (*att*) phenotypes that are specified by mutations in the internal genes. Reassortant H1N1 and H3N2 human influenza

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A viruses with the six internal gene segments of the AA ca virus bear these phenotypes and extensive evaluation in humans has proven them to be attenuated and safe (Vesikari et al., 2006). Accordingly, they have been approved for use as live virus vaccines in humans. Here, we generated a recombinant H1N1 virus that bears the HA and NA genes of a 2009 H1N1 virus isolated in China and the six internal genes from the cold-adapted attenuated virus A/Ann Arbor/6/60 (AA*ca*). We evaluated its efficacy against homologous 2009 pandemic virus, heterologous H1N1 virus, and H5N1 influenza viruses in mice.

2. Materials and methods

2.1. Viruses

H1N1 virus A/Sichuan/1/2009 (SC/09) was isolated from the first human case of the 2009 influenza pandemic in China (Xu et al., 2011). A/Tianjin/15/2009 (H1N1; TJ/09), a representative strain of previously seasonal influenza virus, was isolated from a patient in 2009. H5N1 cold-adapted reassortant virus AH/AA*ca* was constructed by using reverse genetics as previously reported (Fan et al., 2009). The H5N1 virus A/Anhui/2/2005 (AH/05) was isolated from the tracheal secretions of a patient from Anhui province (China) who suffered a lethal outcome in 2005 (Li et al., 2010; Shu et al., 2006; Yu et al., 2007). Virus stocks were propagated in specific pathogen-free (SPF) chicken eggs.

2.2. Laboratory facility

All experiments were conducted using biosafety level (BSL) 3+ containment procedures. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. All animal studies were approved by the Review Board of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.3. Generation of the reassortant virus

The reassortant virus containing the HA and NA genes of the SC/09 virus and the internal genes of the AA*ca* virus was generated by use of conventional recombinant methodology as previously described (Chen et al., 2003b). Briefly, 100 μl of ultraviolet light-treated SC/09 virus and 50 μl of the AH/AA*ca* virus were mixed with 850 μl phosphate-buffered saline (PBS), and the mixture was injected into the allantoic cavity of 10-day-old chicken embryonated eggs. The allantoic fluid from the eggs was harvested after a 24-h incubation at 35 °C. To select for viruses that lacked the HA and NA of the AH/AA*ca* donor virus, 50 μl of fluid was mixed with 600 μl of serially (fourfold) diluted anti-H5N1 SPF chicken antiserum and incubated at room temperature for 30 min prior to being injected into eggs. Allantoic fluid was harvested after a 48-h incubation at 35 °C and tested for evidence of hemagglutination. The sample with hemagglutinating activity was biologically cloned three times by limiting dilution in chicken embryonated eggs. At each passage, the genotype of the virus was determined by use of reverse transcription-polymerase chain reaction (RT-PCR) with strain- and segment-specific primers (primer sequences available upon request). The resulting virus, SC/AA*ca,* was confirmed by means of sequence analysis.

2.4. Phenotypic analysis of the ca reassortant viruses

The *ca* and *ts* phenotypes of SC/AA*ca* were determined as previously described (Chen et al., 2003a). To test the attenuation phenotype of the viruses, groups of 12 mice were anesthetized with CO_2 and inoculated intranasally with 10^6 EID_{50} of the test viruses. Three mice per group were killed on days 1, 3, 5, and 7 post-inoculation (p.i.) and their nasal turbinates and lungs were collected for virus titration in eggs.

2.5. Mouse study

Six-week-old female specific-pathogen-free BALB/c mice were used in this study. To determine the vaccination dosage required to induce protective immunity, groups of five mice were anesthetized with CO_2 before being vaccinated intranasally with different doses (10^4–10^8 EID_{50}) of the virus. Three weeks post-vaccination (p.v.), sera were collected for hemagglutinin inhibition (HI) antibody detection. The mice were then challenged with 100-fold 50% mouse infectious dose (MID_{50}) of the SC/09 virus. On day 4 post-challenge (p.c.), the mice were killed and their nasal turbinates and lungs were collected for virus titration in eggs.

To evaluate the protective efficacy of the SC/AA*ca* virus against the heterologous H1N1 influenza and H5N1 avian influenza viruses, four groups of 11 mice were inoculated with 10^7 EID_{50} of SC/AA*ca* or PBS intranasally and challenged 3 weeks p.v. with 100 MID_{50} of TJ/09 or 100 50% mouse lethal dose (MLD_{50}) of AH/05 ($10^{3.5}$ EID_{50}) virus intranasally. Three mice per group were euthanized on days 4 and 6 p.c. and organs were collected for virus titration in eggs, the remaining five mice were observed daily for weight loss or death for two weeks.

2.6. Detection of antibodies

Sera were treated with *Vibrio* cholera (Denka-Seiken, www.denka-seiken.co.jp) receptor-destroying enzyme before being tested for the presence of HI antibody with 0.5% (V/V) chicken erythrocytes. The neutralization (NT) antibody titers were determined in eggs with heat-inactivated sera collected from mice. HI and NT antibody titers were transformed into $\log_{10}$ titers for the calculation of mean ± S.D. values.

2.7. Bone marrow-derived dendritic cell (BMDC) preparation and stimulation

Mouse BMDCs were generated by following the procedures of Lutz et al. (1999). Briefly, femurs were obtained from sacrificed mice and the marrows were flushed out with complete medium (RPM1640 plus 10% fetal calf serum) and then filtered through a cell strainer. After the red blood cells were lysed, 2×10^6 bone marrow cells were cultured in a 100 mm bacterial petri-dish in 10 ml of culture medium (complete medium supplemented with 20 ng/ml of recombinant mouse GM-CSF), (Peprotech, Rocky Hill, NJ). Three days later, another 10 ml of complete medium was added. Half of the medium was replaced with fresh culture medium on days 6 and 8. For complete maturation, on day 10, non-adherent cells were collected and resuspended in 10 ml of fresh complete medium in a dish containing 20 ng/ml mouse GM-CSF and 1 μg/ml lipopolysaccharide (LPS, Sigma) and then cultured for one more day. Over 70% of these cultured cells should be CD11c-positive based on flow cytometry analysis. BMDCs were infected with influenza virus at a multiplicity of infection (MOI) of five and incubated overnight for dendritic cell (DC)-stimulation; 10^5 influenza virus-pulsed BMDCs were then mixed with 10^6 splenocytes for antigen-specific T cell stimulation.

2.8. Flow cytometry analysis of CD4+ and CD8+ T cell responses

Groups of ten 6-weeks-old female BALB/c mice were infected intranasally with 10^6 EID_{50} of SC/AA*ca*, SC/09, or PBS as a control.

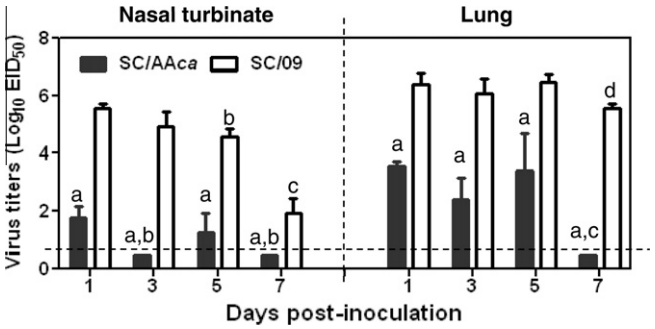

Fig. 1. Replication of the SC/AA*ca* reassortant and SC/09 virus in the respiratory tract of mice. Groups of 6-weeks-old female BALB/c mice were intranasally infected with 10^6 EID_{50} of the test viruses. Three mice in each group were killed on days 1, 3, 5, and 7 post-infection (p.i.) and their nasal turbinates and lungs collected for virus titration in eggs. Virus titers are expressed as the mean ± standard deviation (S.D.) $\log_{10}$ EID_{50}/ml. Samples from which virus was not detected in 0.1 ml of organ homogenate were assigned the numeric value of 0.5 for calculation purposes. a, $P < 0.01$ compared with the corresponding value for the SC/09-inoculated group; b, $P < 0.01$ compared with the corresponding value on day 1 p.i.; c, $P < 0.01$ compared with the corresponding value on day 1, 3, and 5 p.i.; d, $P < 0.01$ compared with the corresponding value on day 1, and 5 p.i. The dashed line indicates the limit of detection.

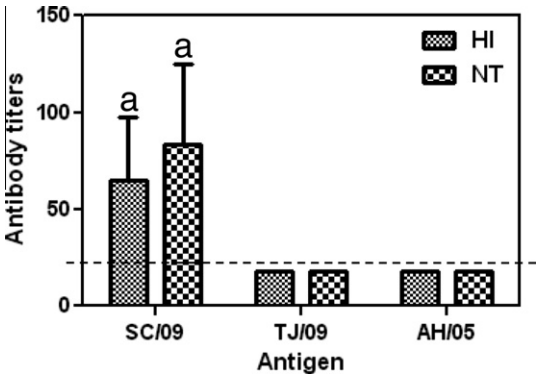

Fig. 3. Antibody response to homologous and heterologous influenza viruses induced by the SC/AAca virus in mice. Twenty-two mice were vaccinated with 10^7 EID_{50} of SC/AA*ca* intranasally, and sera were collected randomly from 10 animals for detection of HI and NT antibodies to different viruses at three weeks p.v. Data shown are the mean ± S.D. Negative results were assigned the numeric value of 10 for calculation purposes. (a) $P < 0.01$ compared with the corresponding value acquired by using TJ/09 and AH/05 as antigens. The dashed line indicates the limit of detection.

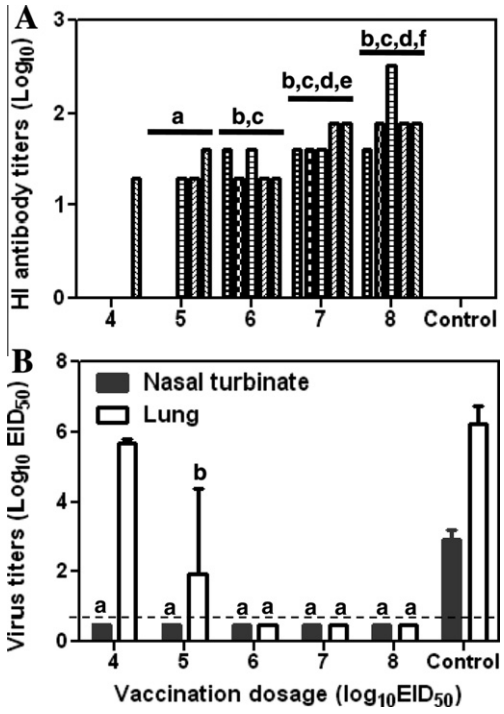

Fig. 2. Protective efficacy against SC/09 challenge in mice inoculated with different dosages of SC/AA*ca*. Groups of five mice were vaccinated intranasally with the indicated dosage of the SC/AA*ca* virus. Three weeks post-vaccination (p.v.), sera were collected for HI antibody detection, and then the mice were challenged with 100-fold MID_{50} of the SC/09 virus. Mice were killed on day 4 post-challenge (p.c.) and their nasal turbinates and lungs were collected for virus titration in eggs. (A) HI antibody titers, each bar indicates the titer from an individual mouse. (a) $P < 0.05$ compared with the corresponding value for the PBS-inoculated group; (b) $P < 0.01$ compared with the corresponding value for the PBS-inoculated group; (c) $P < 0.01$ compared with the corresponding value for the $4\log_{10}$ EID_{50}-inoculated group; (d) $P < 0.01$ compared with the corresponding value for the $5\log_{10}$ EID_{50}-inoculated group; (e) $P < 0.05$ compared with the corresponding value for the $6\log_{10}$ EID_{50}-inoculated group; (f) $P < 0.01$ compared with the corresponding value for the $6\log_{10}$ EID_{50}-inoculated group. (B) Viral titers in nasal turbinates and lungs of mice. Virus titers are expressed as the mean ± S.D. $\log_{10}$ EID_{50}/ml. Samples from which the virus was not detected in 0.1 ml of organ homogenate were assigned the numeric value of 0.5 for calculation purposes. a, $P < 0.01$ compared with the corresponding value for the PBS-inoculated group and $4\log_{10}$ EID_{50}-inoculated group; b, $P < 0.05$ compared with the corresponding value for the PBS-inoculated group and $4\log_{10}$ EID_{50}-inoculated group. The dashed line indicates the limit of detection.

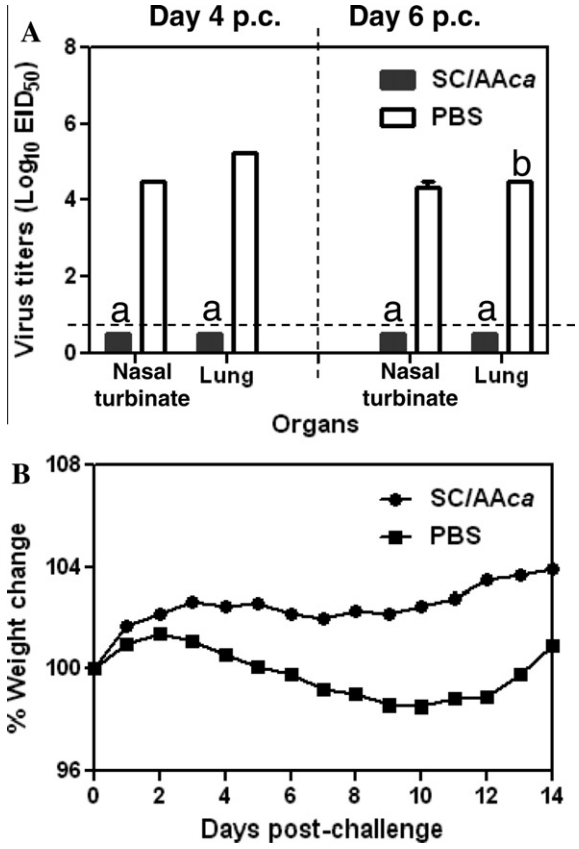

Fig. 4. Protective efficacy of SC/AA*ca* against TJ/09 challenge in mice. Groups of 11 mice were vaccinated with 10^7 EID_{50} of SC/AA*ca* intranasally, and challenged with 100-fold MID_{50} of TJ virus at three weeks p.v. Organs were collected from the three mice that were euthanized on day 4 and day 6 p.c. for virus titration in eggs. Body weights of the remaining five mice were monitored daily for 2 weeks. (A) Challenge virus replication in nasal turbinates and lungs of mice. Virus titers are expressed as the mean ± S.D. $\log_{10}$ EID_{50}/ml. Samples from which the virus was not detected in 0.1 ml of organ homogenate were assigned the numeric value of 0.5 for calculation purposes. The dashed line indicates the limit of detection. (a) $P < 0.01$ compared with the corresponding value for the PBS-inoculated group; (b) $P < 0.01$ compared with the corresponding value on day 4 p.c. (B) Weight changes in mice after challenge with the TJ/09 virus.

Five mice from each group were sacrificed on day 10 p.i. and 3 weeks p.i., respectively. Splenocytes were prepared as described by Ye et al. (2006). Briefly, the spleen was removed from the euthanized mice, cut into small pieces, and then homogenized by gently rubbing. After low speed centrifugation, the supernatant was removed and the cells were gently re-suspended in red blood cell lysing buffer (Sigma) and incubated in ice-water for one minute. The splenocytes were then filtered through a cell strainer and seeded in a 96-well plate (10^6 cells/well). Lymphocytes were stimulated with 1×10^5 influenza virus-pulsed BMDCs for CD4+ and CD8+ T cell responses or with 20 μg/ml of conserved peptides corresponding to the HA (IYSTVASSL) (Kuwano et al., 1988) or NP (TYQRTRALV) (Rotzschke et al., 1990) of SC/09, TJ/09, and AH/05 for 6 h in the presence of 10 ng/ml Brefeldin A (eBioscience, San Diego, CA) for CD8+ T cell responses. After stimulation, the cells were washed twice with PBS containing 3% fetal calf serum and then stained with FITC-conjugated rat anti-mouse CD4 and phycoerythrin (PE)-conjugated rat anti-mouse CD8 antibodies (BD Pharmingen, San Diego, CA). Cells were fixed and permeabilized with Fix & Perm buffers and stained for intracellular interferon gamma (IFN-γ) with an allophycocyanin (APC)-conjugated rat anti-mouse IFN-γ antibody (BD Pharmingen, San Diego, CA). The levels of CD4+ and CD8+ T cell responses were determined by using flow cytometry on a BD FACSAria Station (BD Immunocytometry Systems, San Jose, CA). Data were analyzed with FCS Express software (De Novo Software, Los Angeles, CA).

2.9. Statistical analysis

Viral titers, antibody titers, and the T cell response of mice were compared by use of the two-sided *t*-test. Viral titers between the two different viruses inoculated groups or between the vaccinated group and the PBS group were compared, and the viral titers for each organ that was collected at different time points were also compared. The HI antibody titers of mice inoculated with different doses of the SC/AA*ca* virus were compared with the control group or with each other. The CD4+ and CD8+ T cell responses of mice induced by the SC/AA*ca* and SC/09 virus were compared with those in the PBS control group and with each other, and the data generated from different time points were also compared.

3. Results

3.1. Characterization of the reassortant SC/AAca virus

A reassortant virus, SC/AA*ca*, which derived its HA and NA genes from the SC/09 virus and its six internal genes from the AH/AA*ca* virus, was generated by co-infecting SPF eggs with the two parent viruses. The authenticity of expected genotype of the virus was confirmed by use of sequence analysis. The *ca* and *ts* phenotypes of SC/AA*ca*, specified by the internal genes, were confirmed in eggs and tissue culture, respectively (data not shown).

We then evaluated the replication of the viruses in mice. Two groups of 12 mice were inoculated i.n. with 10^6 EID_{50} of SC/09 and SC/AA*ca*, respectively. Three mice in each group were euthanized on days 1, 3, 5 and 7 post inoculation (p.i.) and nasal turbinates and lungs of the mice were collected for virus titration. Viruses were detected in the mice inoculated with SC/09 at all four time points p.i., whereas virus was detected in the mice inoculated with SC/AA*ca* on days 1, 3 and 5 p.i., but not on day 7 p.i. Moreover these titers were significantly lower than those in mice inoculated with SC/09 (Fig. 1).

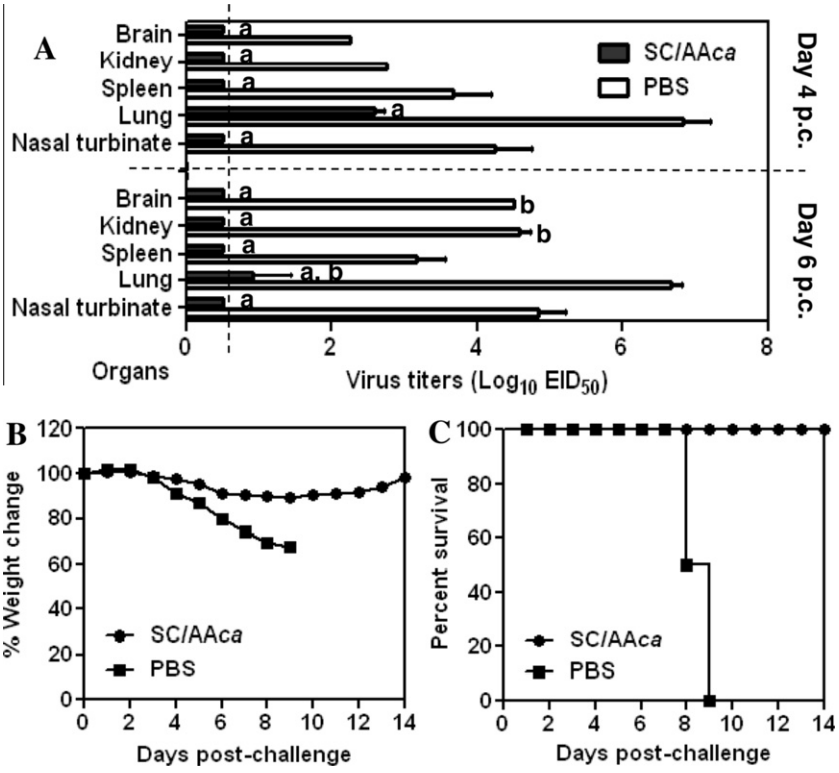

Fig. 5. Protective efficacy of SC/AA*ca* against H5N1 AH/05 challenge in mice. Groups of 11 mice were vaccinated with 10^7 EID_{50} of SC/AA*ca* intranasally, and challenged with 100-fold MLD_{50} of AH/05 virus at three weeks p.v. Organs were collected from the three mice that were euthanized on day 4 and day 6 p.c. for virus titration in eggs. Body weights and survival of the remaining five mice were monitored daily for 2 weeks. (A) Challenge virus replication in organs of mice. Virus titers are expressed as the mean ± S.D. $\log_{10}$ EID_{50}/ml. Samples from which the virus was not detected in 0.1 ml of organ homogenate were assigned the numeric value of 0.5 for calculation purposes. The dashed line indicates the limit of detection. (a) $P < 0.01$ compared with the corresponding value for the PBS-inoculated group; (b) $P < 0.01$ compared with the corresponding value on day 4 p.c. (B) Weight changes in mice after challenge with the AH/05 virus. (C) Death rates of mice after challenge with AH/05 virus.

3.2. Protective efficacy of SC/AAca against the pandemic H1N1 virus in mice

To evaluate the immunogenicity of the SC/AA*ca* virus, groups of 6-week-old BALB/c mice were inoculated with different dosages (10^4–10^8 EID_{50}) of SC/AA*ca*. The HI antibody titers in these animals are shown in Fig. 2A. The HI antibody titers in all of the SC/AA*ca*-inoculated groups, except for the 4 $\log_{10}$ EID_{50} SC/AA*ca*-inoculated group, were significantly higher than that in the PBS-inoculated groups (Fig. 2A). There was no significant difference in HI antibody titers between the 4–5 $\log_{10}$ EID_{50} SC/AA*ca*-inoculated groups, although the numbers of HI antibody-positive animals in these two groups were different. The HI antibody titers in the 6–8 $\log_{10}$ EID_{50} SC/AA*ca*-inoculated groups were significantly higher than that in 4 $\log_{10}$ EID_{50} SC/AA*ca*-inoculated group. The HI antibody titers in the 7–8 $\log_{10}$ EID_{50} SC/AA*ca* groups were also significantly higher than those in the 5–6 $\log_{10}$ EID_{50} SC/AA*ca*-inoculated groups, but there was no difference between the titers in the 7–8 $\log_{10}$ EID_{50} SC/AA*ca* groups. These results indicate that there was strong dose–response relationship between the HI antibody titers of the mice and the virus dose inoculated.

The protective efficacy of SC/AA*ca* as a live virus vaccine was evaluated in mice that were subsequently challenged with the SC/09 virus. In the PBS-inoculated group, the challenge SC/09 virus replicated to mean titers of 2.9 $\log_{10}$ EID_{50} in the NTs and 6.3 $\log_{10}$ EID_{50} in the lungs (Fig. 2B). In contrast, the challenge SC/09 virus was not detected in the NTs of any immunized mice, although the challenge SC/09 virus was detected in the lung of mice immunized with 10^4 or 10^5 EID_{50} of SC/AA*ca*, the titers in the 10^5 EID_{50} of SC/AA*ca*-inoculated group were significantly lower than those detected in the PBS-inoculated mice (Fig. 2B). These results indicate that SC/AA*ca* is immunogenic and one dose of 10^6 EID_{50} or higher provides complete protection against homologous SC/09 virus challenge.

3.3. Protective efficacy of SC/AAca against seasonal H1N1 virus and H5N1 virus challenge in mice

Mice were inoculated with 10^7 EID_{50} of SC/AA*ca* and three weeks later sera were collected to test for HI and NT antibodies against H1N1 and H5N1 viruses. Although mice developed HI and NT antibody against the homologous SC/09 virus, there were no detectable antibodies against the TJ/09 and AH/05 viruses (Fig. 3).

To evaluate the protective efficacy of SC/AA*ca* against seasonal H1N1 virus, mice were challenged with 100 MID_{50} of the TJ/09 virus. Replication of the challenge virus was completely prevented in the vaccinated mice, whereas high titers of virus, ranging from 4–6 $\log_{10}$ EID_{50}, were detected in the nasal turbinates and lungs of control mice on days 4 and 6 p.c. (Fig. 4A). The body weight of the vaccinated mice steadily increased relative to that of the control mice (Fig. 4B).

We then evaluated the protective efficacy of SC/AA*ca* against the H5N1 virus. In the SC/AA*ca*-vaccinated group, replication of the H5N1 challenge virus was only detected in the lung of the mice at both time points tested, and the titers at day 6 p.c. was significantly lower than those at day 4 p.c. The titers of challenge virus in the lung of SC/AA*ca*-vaccinated group were significantly lower than those in the control mice ($P < 0.01$). All of the vaccinated mice remained healthy and survived the two-week observation period (Fig. 5B, C). However, in the PBS-inoculated control mice, high titers of challenge virus were detected in all of the organs tested on both day 4 and day 6 p.c. (Fig. 5A), and the viral titers in the brain and kidney of mice at day 6 p.c. were significantly higher than those at day 4 p.c.; all of these mice lost body weight and died within ten days of challenge (Fig. 5B, C).

3.4. Cellular immune response

Mice vaccinated with SC/AA*ca* did not develop detectable cross-reactive HI and NT antibodies against the TJ/09 and AH/05 viruses; however, they were protected from challenge with these two viruses. To understand the underlying mechanism, the specific CD4+ and CD8+ T cell responses of mice inoculated with wild-type SC/09 or SC/AA*ca* virus were evaluated at both 10 days p.i. and 3 weeks p.i. At day 10 p.i., mice infected with SC/AA*ca* virus had a significant CD4+ T cell response to SC/09 ($P < 0.01$) and TJ/09 ($P < 0.01$) viruses relative to that in the PBS-inoculated group, but had no significant response to AH/05 virus (Fig. 6A). However, mice infected with SC/09 virus had a significant CD4+ T cell response to SC/09 ($P < 0.01$), TJ/09 ($P < 0.01$), and AH/05 ($P < 0.05$) viruses relative to that in the PBS inoculated group, and their CD4+ T cell responses to SC/09 and TJ/09 were significantly higher than those to AH/05 ($P < 0.05$) (Fig. 6A). At 3 weeks p.i., mice infected with SC/AA*ca* virus had a significant CD4+ T cell response to SC/09 ($P < 0.05$) and AH/05 ($P < 0.01$) viruses relative to that in the PBS-inoculated group ($P < 0.05$), but no significant response to TJ/09 virus. However, mice infected with SC/09 virus had a significant CD4+ T cell response to SC/09 ($P < 0.01$), TJ/09 ($P < 0.05$) and AH/05 ($P < 0.01$) viruses relative to those in the PBS-inoculated group. No differences were detected among the different viral antigen-stimulated groups. In both the SC/AA*ca* and SC/09 inoculated mice,

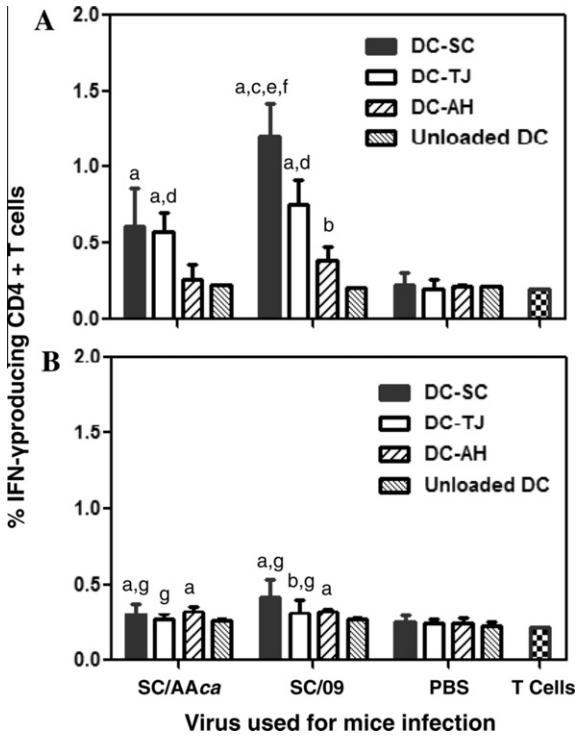

Fig. 6. CD4+ T cell responses to different influenza viruses in mice inoculated with SC/AA*ca*. Groups of five 6-weeks-old female BALB/c mice were intranasally infected with 10^6 EID_{50} of SC/AA*ca*, SC/09, or PBS as a control. The interferon-γ-producing CD4+ T cells against different antigens were tested from the samples collected at day 10 p.i. (A) or at 3 weeks p.i. (B) as described in the Materials and methods. The data shown are the mean ± S.D. for each group. (a) $P < 0.01$ compared with the corresponding value for the PBS-inoculated group, T cell group, or the value for the unloaded DC group; (b) $P < 0.05$ compared with the corresponding value for the PBS-inoculated group, T cell group, or the value for the unloaded DC group; (c) $P < 0.01$ compared with the corresponding value for the SC/AA*ca*-inoculated group; (d) $P < 0.05$ compared with the corresponding value for the AH/05-stimulated group (DC–AH); (e) $P < 0.01$ compared with the corresponding value for the AH/05-stimulated group (DC–AH); (f) $P < 0.05$ compared with the corresponding value for the TJ/09-stimulated group (DC–TJ); (g) $P < 0.01$ compared with the corresponding value at day 10 p.i.

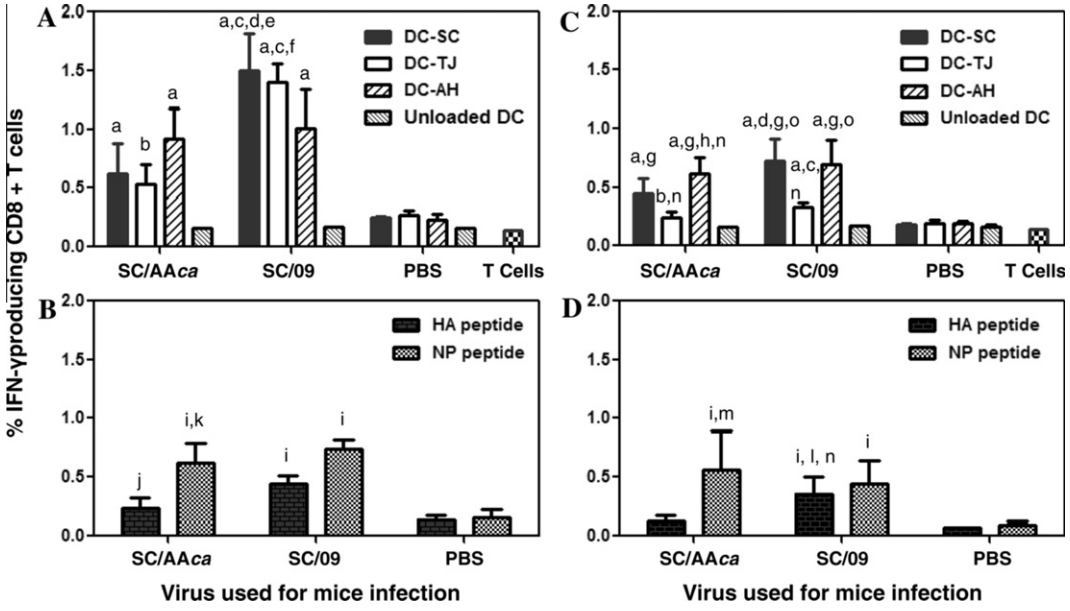

Fig. 7. CD8+ T cell responses in mice inoculated with SC/AA*ca*. Groups of five 6-weeks-old female BALB/c mice were intranasally infected with 10^6 EID_{50} of SC/AA*ca*, SC/09, or PBS as a control. Interferon-γ-producing CD8+ T cells against different viral antigens (A, C) or peptides (B, D) were determined from samples that were collected at day 10 p.i. (A, B) and 3 weeks p.i. (C, D). The data shown are the mean ± S.D. for each group. (a) $P < 0.01$ compared with the corresponding value for the PBS-inoculated group, T cell group, or the value for the unloaded DC group; (b) $P < 0.05$ compared with the corresponding value for the PBS-inoculated group, T cell group, or the value for the unloaded DC group; (c) $P < 0.01$ compared with the corresponding value for the SC/AA*ca*-inoculated group; (d) $P < 0.05$ compared with the corresponding value for the TJ/09-stimulated group (DC–TJ); (e) $P < 0.01$ compared with the corresponding value for the AH/05-stimulated group (DC–AH); (f) $P < 0.05$ compared with the corresponding value for the AH/05-stimulated group (DC–AH); (g) $P < 0.01$ compared with the corresponding value for the TJ/09-stimulated group (DC–TJ); (h) $P < 0.05$ compared with the corresponding value for the SC/09-stimulated group (DC–SC); (i) $P < 0.01$ compared with the corresponding value for the PBS group; (j) $P < 0.05$ compared with the corresponding value for the PBS group; (k) $P < 0.05$ compared with the corresponding value for the HA peptide-stimulated group (HA peptide); (l) $P < 0.01$ compared with the corresponding value for the SC/AA*ca* group; (m) $P < 0.01$ compared with the corresponding value for the HA peptide-stimulated group (HA peptide); (n) $P < 0.05$ compared with the corresponding value on day 10 p.i.; (o) $P < 0.01$ compared with the corresponding value on day 10 p.i.

the CD4+ T cell response to SC/09 and TJ/09 at 3 weeks were significantly lower than that at 10 days p.i., but their response to the AH/05 virus at these two time points were comparable (Fig. 6B).

CD8+ T cell responses to SC/09, TJ/09, and AH/05 viruses were detected in the SC/AA*ca* virus- and SC/09 virus-infected mice at both 10 days p.i. and 3 weeks p.i. (Fig. 7A, B). At day 10 p.i., the CD8+ T cell responses to SC/09 virus and TJ/09 virus in the SC/09-inoculated mice were significantly higher than that in the SC/AA*ca*-inoculated mice ($P < 0.01$). The CD8+ T cell responses to SC/09 virus and AH/05 virus were significantly higher than that to the TJ/09 virus in both the SC/AA*ca*- and SC/09-infected mice at 3 weeks p.i. (Fig. 7C), although the responses in both virus-inoculated mice to all these three viruses were significantly lower than that at 10 days p.i. The CD8+ T cell responses were detected after stimulation with the HA and NP peptides in both the SC/AA*ca*- and SC/09-inoculated mice (Fig. 7B, D); the response to the NP peptide was significantly higher than that to the HA peptide (Fig. 7B, D). These results indicate that both the vaccine strain SC/AA*ca* and the wild-type SC/09 virus could induce significant cellular immune responses to heterologous H1N1 and H5N1 influenza viruses, suggesting that cellular immunity may play an important role in mice vaccinated with SC/AA*ca* in terms of cross-protection against the TJ/09 and AH/05 viruses.

4. Discussion

Here, we generated an H1N1 reassortant virus (SC/AA*ca*) that exhibits the *ca*, *ts*, and *att* phenotypes of the AA*ca* parent virus and is antigenically similar to the SC/09 parent virus. A single dose of SC/AA*ca* administered i.n. as a live virus vaccine was immunogenic and protected mice against subsequent challenge with homologous and antigenically heterologous H1N1 seasonal influenza virus. Moreover, this live vaccine also provided sound protection against lethal H5N1 virus challenge in mice.

The 2009 H1N1 is the most widely distributed influenza virus in the world since 2009, but seasonal H1N1 influenza viruses can still be detected (CDC, 2010; Ilyicheva et al., 2011; Raboni et al., 2011; Van Kerkhove et al., 2011; Wang et al., 2011). An inactivated vaccine targeted at the 2009 H1N1 virus has been developed and used in many countries (Girard et al., 2010b; Xie et al., 2011). H1N1 cold-adapted live vaccines have also been developed and used in several countries (Belshe, 2004; Dhere et al., 2011; Perdue and Bright, 2011; Rudenko et al., 2011; Schultz-Cherry and Jones, 2010; Tennis et al., 2011). The inactivated vaccine mainly induces a humoral immune response, whereas the live attenuated vaccine induces both humoral and cellular immune responses (Cox et al., 2004). One dose of the SC/AA*ca* vaccine was unable to induce detectable cross-reactive HI and NT antibodies against the TJ/09 virus; however, both CD4+ T cell and CD8+ T cell responses to TJ/09 were detected in the vaccinated mice. Complete protection against TJ/09 virus challenge in mice may, therefore, be largely attributable to the T cell immune responses.

CD4+ T cells play a role in the antibody response against H5N1 influenza virus in monkeys inoculated with live attenuated vaccine (Fan et al., 2009). Here, following SC/AA*ca* inoculation, mice developed good antibody responses against homologous SC/09 virus, but HI and NT antibodies against the heterologous TJ/09 virus were not detectable. However, the CD4+ T cell responses against homologous SC/09 and the heterologous TJ/09 virus did not show a significant difference in the SC/AA*ca*-inoculated mice. These results indicate that the CD4+ T cell epitopes are conserved in the SC/09 and TJ/09 viruses, although the antigenicity of these two viruses, which is determined by the HA protein, was significantly different.

Robust CD8+ T cell responses against the homologous SC/09 and heterologous TJ/09 and AH/05 viruses were detected at both

10 days p.i. and 3 weeks p.i. with the SC/AA*ca* virus and SC/09 virus. However, the CD4+ T cell response against the SC/09 and TJ/09 viruses in both the SC/AA*ca*- and SC/09-inoculated mice was significantly reduced at 3 weeks p.i. compared with that at 10 days p.i. These results are in agreement with observations in nonhuman primates inoculated with seasonal H1N1 influenza virus (Weinfurter et al., 2011). Although the number of CD4+ T cells in the spleens of mice was relatively small at 3 weeks p.i., these cells may still have been able to provide several functions to augment the antiviral immune response, by providing "help" to CD8+ T cells and B cells, and perhaps most importantly by recruiting immune cells to sites of virus replication.

The H5N1 influenza viruses have been detected in wild birds and domestic poultry in more than 60 countries around the world and are still circulating in several countries, posing a huge pandemic threat. Although different H5N1 pandemic vaccines have been developed and tested in humans and animals (Fedson, 2005; Vajo et al., 2010), none of them will be used in humans before the pandemic occurs. We found that our H1N1 live attenuated vaccine SC/AA*ca* protected mice from lethal H5N1 virus challenge, restricting the replication of the challenged virus to the lung and significantly reducing virus titers. This result is in agreement with the report that inoculation of mice with the AA*ca* virus confers partial protection against H5N1 virus challenge (Suguitan et al., 2006). We further demonstrated that the SC/AA*ca* and wild-type SC/09 viruses induced CD4+ and CD8+ T cell responses against the H5N1 AH/05 virus, which may have contributed to the observed protection against the challenge virus. These results imply that inoculation with the 2009 H1N1 live vaccine or pre-infection with the 2009 H1N1 wild-type virus may reduce the severity of H5N1 virus infections in humans, although this pre-immunity may not be able to prevent heterologous virus infection due to a lack of antibodies, which play a key role in preventing virus infection. Indeed, previous human studies have confirmed that the cross-reactive T cell responses elicited after natural influenza virus infections reduce the viral replication and decrease disease severity caused by subsequent heterologous influenza virus challenge (McMichael et al., 1983).

In summary, we generated a live attenuated virus, SC/AA*ca*, and found that the inoculation of this virus provided sound protection against homologous 2009 pandemic H1N1 virus, heterologous seasonal H1N1 virus and H5N1 virus challenge in mice. Although SC/AA*ca* inoculation did not induce any cross-reactive antibodies against the heterologous H1N1 and H5N1 viruses, both CD4+ T cell and CD8+ T cell responses against the heterologous H1N1 and H5N1 viruses were detected in mice, indicating that T cell responses play an important role in protection against heterologous virus challenge in this animal model. Our study also suggests that the induction of cross-reactive virus-specific T cell responses may be an effective approach for the development of universal influenza vaccines.

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