High immune efficacy against different avian influenza H5N1 viruses due to oral administration of a *Saccharomyces cerevisiae*-based vaccine in chickens

Han Lei*, Xin Lu, Shuangqin Li & Yi Ren

A safe and effective vaccine is the best way to control large-scale highly pathogenic avian influenza virus (HPAI) A (H5N1) outbreaks. *Saccharomyces cerevisiae* (*S. cerevisiae*) is an ideal mucosal delivery vector for vaccine development, and we have previously shown that conventional administration of a *S. cerevisiae*-based vaccine (EBY100/pYD1-HA) via injection led to protection against the homologous H5N1 virus in a mouse model. Because the diameter of *S. cerevisiae* is approximately 10 μm, which results in a severe inflammation by injection route, therefore, oral administration is a more suitable approach for EBY100/pYD1-HA conferring protection in poultry. We extended our work by evaluating the immunogenicity and protective efficacy of oral vaccination with EBY100/pYD1-HA in the chicken model. Oral immunization with EBY100/pYD1-HA could induce robust serum IgG, mucosal IgA and cellular immune responses. Importantly, EBY100/pYD1-HA provided protection against challenges with a homologous and a heterologous H5N1 viruses. These findings suggest that EBY100/pYD1-HA, a promising H5N1 oral vaccine candidate, can avoid potential reassortment of other avian influenza viruses in oral administration of live virus vaccines and overcome the limitations of conventional injection routes. Importantly, this platform will be able to provide opportunities for broader applications in poultry during HPAI A (H5N1) outbreaks.

The emergence and spread of highly pathogenic avian influenza (HPAI) A (H5N1) viruses have fueled concerns of a potential zoonotic pandemic originating in poultry, and spurred efforts towards developing vaccines against A (H5N1) influenza viruses and improving vaccine production methods. The current licensed vaccines, including adjuvanted formulations, predominately include inactivated whole avian influenza H5N1 and are available for the control of outbreaks in poultry. These vaccines have limitations since they require intramuscular injection, and the biosecurity of these vaccines has not been fully elucidated. In addition, some of these vaccines were poorly immunogenic and may require a higher concentration of the immunogen to achieve protective immunogenicity. Further, the egg-based manufacturing processes of these vaccines also have safety and production issues. A live-attenuated A (H5N1) vaccine has been generated by reverse genetics, but the risk of virus reassortment in the field prohibits the use of this vaccine in most instances. Thus, there is a clear need for new vaccine formulations and delivery strategies that can provide increased efficacy and safety.

In attempts to develop more efficacious A(H5N1) vaccines, several formulations, such as mammalian cell-based vaccines, recombinant protein-based vaccines, recombinant virus-like particle (VLP) vaccines, DNA vaccines, bacteria- or yeast-vectored vaccines, and viral-vectored vaccines, have been extensively explored as alternative approaches. Included in the list of alternative strategies are the recombinant yeast-based A (H5N1) vaccines, which are promising candidates that meet the requirement of vaccine production in a timely manner and can induce robust protective immunity against A(H5N1) virus infection.

*Saccharomyces cerevisiae* is a representative strain of yeast and is widely used in industries performing fermentation, particularly for the food industry. As a novel strategy in the fight against infectious diseases, S.
cerevisiae-based vaccines hold great promise for both public health and domestic poultry. Mucosal delivery of vaccine is superior to conventional methods of injection in terms of operative ease and safety. Oral administration is an economical approach for enhancing mucosal immunity in order to control influenza virus infection while reducing the cost of vaccine delivery. Furthermore, compared to the intracellular expression of recombinant viral proteins, the display of viral proteins on the carrier cell surface can facilitate their recognition by the host mucosal immune system, thereby enhancing their capability of eliciting protective immunity.

We have previously shown that EBY100/pYD1-HA could provide protection when administered by injection route in a mouse model. However, EBY100/pYD1-HA induces serious inflammation at the injection site due to the diameter of S. cerevisiae is approximately 10 μm. Furthermore, chickens are the primary model for studies of pathogenicity and vaccine efficacy studies for poultry. To address this issue, we hypothesize that oral vaccination with unadjuvanted EBY100/pYD1-HA can produce protective immunity in the chicken model and can be considered an effective platform for the development of an influenza A (H5N1) vaccine for the mass vaccination of poultry.

In the present study, we extended our previous work by evaluating the immunogenicity of EBY100/pYD1-HA in a chicken model. Oral vaccination with EBY100/pYD1-HA induces strong humoral, cell-mediated and mucosal immunity and confers protection against challenges with a homologous and a heterologous H5N1 viruses. Importantly, the production of EBY100/pYD1-HA just requires 2 weeks, and thus, the vaccine has great potential for mass production in a short period of time for use in poultry during influenza A (H5N1) outbreaks.

**Results**

Expression and quantification of EBY100/pYD1-HA. Western blot analysis was performed to determine the expression of HA protein, the expected band corresponding to 75 kDa was observed in the lysates of EBY100/pYD1-HA (Fig. 1a, Lane 1), which consisted of Aga2 (10 kDa) and HA protein (65 kDa), whereas it was absent in the lysates of EBY100/pYD1 (Fig. 1a, Lane 2).

As shown in Fig. 1b, it was found that the concentration of the displayed HA protein was approximately 60 μg/mL on the cell surface of S. cerevisiae (Fig. 1b), when increasing concentration of monoclonal anti-HA antibody was used against 5 OD600nm of EBY100/pYD1-HA. When the concentration of antibody was increased beyond this point, the optical density was relatively stable, which suggested that 5 OD600nm of EBY100/pYD1-HA expressing HA protein was at its saturation limit at 60 μg/mL compared with the known concentration of purified HA protein.

**Determination of HA-specific antibody responses.** To evaluate the antibody responses induced by EBY100/pYD1-HA, the IgG levels in the serum and the IgA levels in the intestine washes were separately measured by ELISA on days 13 and 28 after the initial vaccination. The group that received EBY100/pYD1-HA was able to respond with effective and significant HA-specific serum IgG (Fig. 2a) and mucosal IgA antibody (Fig. 2b) levels compared to control groups (PBS and EBY100/pYD1). Therefore, these results indicate that oral
administration of EBY100/pYD1-HA can induce robust humoral and mucosal immune responses in a chicken model.

**Cellular immune responses induced by EBY100/pYD1-HA.** To further examine the cellular immunity induced by EBY100/pYD1-HA, we assessed IFN-γ and IL-4-secreting splenocytes using ELISpot kits. Splenocytes were isolated from the vaccinated chickens on days 13 and 28 after the initial immunization and stimulated with a HA-specific peptide. The levels of IFN-γ and IL-4-secreting cells in the EBY100/pYD1-HA group were significantly higher than those in the control groups (Fig. 3). The levels of IFN-γ-secreting cells were higher than the levels of IL-4-secreting cells in the EBY100/pYD1-HA group (Fig. 3). Taken together, these results demonstrate that EBY100/pYD1-HA can induce both Th1- and Th2-type immune responses, with preference of the Th1 type immune responses, as evidenced by the higher levels of IFN-γ production.

**HI titers.** To assess the induction of functional antibody responses elicited by EBY100/pYD1-HA, serum was collected from the chickens orally administrated with PBS or EBY100/pYD1. Regardless of doses, these chickens showed only background levels of HI titers. However, EBY100/pYD1-HA could elicit meaningful HI titers of 64 and 64 against A/Vietnam/1203/2004 (H5N1) (clade 1) or A/Chicken/Henan/12/2004 (H5N1) (clade 8), respec-
tively, on days 28 (Table 1). Therefore, EBY100/pYD1-HA is immunogenic and elicits higher levels of functional antibody responses than the PBS- and EBY100/pYD1 controls.

Immune protective efficacy induced by EBY100/pYD1-HA. To support the potential of EBY100/pYD1-HA to elicit protective responses against different H5N1 viruses, the vaccinated chickens (n = 16 per group) were challenged with a lethal dose (25 μL of 10⁴ EID₅₀) of a homologous A/Vietnam/1203/2004 (H5N1) (clade 1) or a heterologous A/Chicken/Henan/12/2004 (H5N1) (clade 8) virus 2 weeks after the final immunization. The conditions of the chickens in terms of changes in body weight and survival rate were monitored daily for 14 days. As shown in Fig. 4, the chickens that orally administrated with PBS or EBY100/pYD1 showed clinical symptoms of severe disease including significant morbidity (as indicated by weight loss) at day 5 (Fig. 4a,b) and high viral titers in lung at day 3 after influenza A (H5N1) virus infection (Fig. 4c,d), and these chickens died from lethal infection by 8 days (Fig. 4e,f). In contrast, EBY100/pYD1-HA group presented with slight weight loss (Fig. 4a,b) and statistically lower virus titer in the lungs (Fig. 4c,d), and all of these chickens survived and recovered completely after the challenge (Fig. 4e,f). These results provide reliable evidence that EBY100/pYD1 is an effective immunogen for conferring protective immunity with high efficacy against highly pathogenic H5N1 avian influenza viruses.

Table 1. HI titers. HI titers are representative of three independent experiments. Asterisks represent statistical significance compared with the PBS- and EBY100/pYD1 groups. *p < 0.05, **p < 0.01.

| Groups              | A/Vietnam/1203/2004 (H5N1) | A/chicken/Henan/12/2004 (H5N1) |
|---------------------|---------------------------|-------------------------------|
|                     | Day 13 | Day 28 | Day 13 | Day 28 |
| PBS                 | 4      | 8      | 8      | 8      |
| EBY100/pYD1         | 8      | 8      | 8      | 8      |
| EBY100/pYD1-HA      | 32*    | 64**   | 16*    | 64**   |

Figure 3. Cellular immune responses induced by oral administration of EBY/pYD1-HA. IFN-γ- and IL-4-secreting cells (n = 5 chickens per group) were separately analyzed by ELISpot assay. Asterisks indicate significant difference compared with the PBS- and EBY100/pYD1 controls. *p < 0.05, **p < 0.01.
Figure 4. Immune protection efficacy of EBY/pYD1-HA against different H5N1 viruses. Chickens were intranasally challenged with a lethal dose (25 μL of $10^4$ EID$_{50}$) of H5N1 virus. (a) Weight change as a percentage after A/Vietnam/1203/2004 (H5N1) (clade 1) challenge. (b) Weight change as a percentage after A/Chicken/Henan/12/2004 (H5N1) (clade 8) challenge. (c) Lung viral titers at day 3 post A/Vietnam/1203/2004 (H5N1) (clade 1) challenge. (d) Lung viral titers at day 3 after A/Chicken/Henan/12/2004 (H5N1) (clade 8) challenge. (n = 3 chickens per group). (e) Survival rates after A/Vietnam/1203/2004 (H5N1) (clade 1) challenge. (f) Survival rates after A/Chicken/Henan/12/2004 (H5N1) (clade 8) challenge. (n = 10 per group). The weight change data are presented as the means ± SDs. Asterisks indicate significant difference compared with the PBS- and EBY100/pYD1 controls. (*p < 0.05).
Comparing to serious limitations of the manufacturing platform, the currently available avian influenza H5N1 vaccines are generally poorly immunogenic and have safety concerns. Furthermore, most of influenza A (H5N1) vaccines require administration by intramuscular or subcutaneous injection, which has been shown to be insufficient for generating protective immunity at the mucosal surface. The mucosal delivery route can not only induce effective systemic immune responses but also elicit mucosal immune responses, which are helpful for controlling virus replication in the respiratory tract. As an alternative strategy for vaccine development, an edible nonpathogenic vector that carries the recombinant H5N1 HA protein, such as yeast, provides the potential to prevent and control H5N1 virus infection via oral administration.

The induction of EBY100/pYD1-HA was expressed in yeast nitrogen base (YNB)—casamino acids (CAA) medium (20 g/L galactose, 6.7 g/L YNB without amino acids, 13.61 g/L Na2HPO4, 7.48 g/L NaH2PO4 and 5 g/L casamino acids) at 20 °C for 72 h.

**Materials and methods**

**Animals.** All experimental protocols involving animals were approved by the ethics committee of South-west Jiaotong University (Approval number: 7792). All animal procedures were carried out in accordance with the Guidelines for Use and Care of Experimental Animals in Southwest Jiaotong University. All methods are reported in accordance with ARRIVE guidelines.

Specific pathogen-free (SPF) white Leghorn chickens (7 days old) were purchased from SLC Laboratory Center (Shanghai, China) and housed as 5 chicks per cage (50 cm × 45 cm × 45 cm) in an environmentally controlled house. The chickens were fed a pathogen-free diet and water.

All the virus challenge experiments were performed in enhanced animal biosafety level-3 (BSL-3) facilities.

**Vaccine preparedness, oral immunization and sample collection.** HA gene (1650 bp) of A/Vietnam/1203/2004 (H5N1) (clade 1) (GenBank accession No. EU122404) without the signal and transmembrane region was subcloned into surface expression plasmid pYD1, EBY100/pYD1-HA was generated as previously described, except that EBY100/pYD1-HA expressing HA was not quantified.

The expression of HA protein in *S. cerevisiae* was determined by Western blot analysis as described previously. Briefly, 5 OD600 of EBY100/pYD1-HA pellets were boiled for 10 min with 100 µL of 6× sodium casamino acids at 20 °C for 72 h.
dodecyl sulfate (SDS) loading buffer and then run on a 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio-Rad, Hercules, CA, USA). The gel was transferred to a 0.45 μm nitrocellulose membrane. After blocking with 5% nonfat milk at room temperature for 2 h, the membrane was incubated with a monoclonal chicken anti-HA antibody (1:5000 diluted) (R&D Systems, USA) overnight at 4 °C, and followed by 1:5000 diluted horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (R&D Systems, USA) at room temperature for 1 h. Lastly, the membrane was reacted with the West Pico Chemiluminescent Substrate (Bio-Rad, Hercules, CA, USA) in the dark for 5 min and the blot signal was imaged using Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). Meanwhile, Precision Plus Protein™ WesternC™ (Bio-Rad, Hercules, CA, USA) was used as a protein marker.

EBY100/pYD1-HA was inactivated at 60 °C for 1 h and then used for subsequent oral immunization. The final concentration of EBY100/pYD1-HA was adjusted to 0.5 optical density (OD)_{600 nm}/μL. Phosphate-buffer saline (PBS) and EBY100/pYD1 served as controls.

Three groups of chickens (n = 26 per group) were orally immunized with 200 μL of PBS, 100 OD_{600nm} of EBY100/pYD1 or 100 OD_{600nm} of EBY100/pYD1-HA on day 1 (prime immunization) and day 14 (boost immunization).

Sera (n = 10 per group), intestine washes (n = 5 per group) and spleen (n = 5 per group) were collected from the vaccinated chickens on days 13 and 28 after the initial immunization.

Quantification of EBY100/pYD1-HA expressing HA protein. Quantification of the HA protein was performed by enzyme-link immunosorbsent assay (ELISA) as previously described26. In brief, 5 OD_{600nm} of EBY100/pYD1-HA were resuspended in 100 μL of a solution of monoclonal mouse anti-HA antibody (0, 15, 30, 45, 60, 75, 90, 105, 120 μg/mL) (kindly provided by NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA, USA) in PBS containing 2% bovine serum albumin (BSA) and incubated at room temperature for 2 h. This was followed by incubation with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (1 mg/mL) (R&D Systems, Minneapolis, MN, USA) at room temperature for 1 h. After washing with sterile PBS, the plates were resuspended in 100 μL of the HRP substrate 3,3′,5,5′-tetramethylenblazon (TMB) (R&D Systems, Minneapolis, MN, USA) in the dark for 25 min, and then, 100 μL of 2 mol/l H₂SO₄ was added to stop the reaction. The OD_{450nm} value of the supernatant was measured using a microplate reader. EBY100/pYD1 was used a negative control. A solution of purified HA protein (60 μg/mL) (kindly provided by NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA, USA) was used a positive control.

Measurement of antibody responses. HA-specific serum IgG and mucosal IgA antibody levels were separately determined by ELISA as previously described15. Briefly, 2 μg of recombinant HA protein of A/Vietnam/1203/2004 (H5N1) (clade 1) (kindly provided by NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA, USA) was used as the antigen to coat 96-well ELISA plates overnight at 4 °C. The wells were washed three times with Tris-buffered PBS containing 0.05% Tween 20 (TBS-T) and blocked with TBS-T containing 1% BSA at room temperature for 2 h. Sera or intestine washes were added to the plates and incubated at 37 °C for 1 h, followed by incubation with biotinylated goat anti-chicken IgG (R&D Systems, Minneapolis, MN, USA) or biotinylated goat anti–chicken IgA (R&D Systems, Minneapolis, MN, USA) and alkaline phosphatase (AP)-labeled streptavidin (R&D Systems, Minneapolis, MN, USA) at 37 °C for 1 h, respectively. The plates were washed three times with TBS-T and then incubated with 100 μL of p-nitrophenyl phosphate (PNPP) substrate (R&D Systems, Minneapolis, MN, USA). The reaction was developed at room temperature for 25 min and then was stopped with 50 μL of 2 M sodium hydroxide (NaOH). The OD value was measured at 405 nm using an ELISA plate reader (Bio-Tek Instrument Inc., Winooski, USA). The OD_{405nm} of naive controls plus 2 standard deviations.

ELISpot to determine T cell responses. Splenocytes (n = 5 per group) were isolated from the vaccinated chickens on days 13 and 28 after the initial vaccination. HA-specific cells secreting Interferon gamma (IFN-γ) and Interleukin-4 (IL-4) were analyzed using commercial ELISpot assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Briefly, splenocytes (1.0 × 10⁶ cells/well) isolated from the vaccinated chickens were cultured in 96-well plates containing chicken IFN-γ or IL-4 and stimulated with 10 μg/mL of HA–specific peptide (JSVGSTLQNQLVP) for 36 h in a humidified incubator at 37 °C with 5% CO₂. The plates were washed with sterile PBS and incubated with biotinylated goat anti-chicken IFN-γ or IL-4 antibodies overnight at 4 °C, followed by AP-conjugated streptavidin at room temperature for 2 h. The plates were washed, developed with BCIP/NBT, and counted using an ImmunoSpot ELISpot reader (Bio-Tek Instrument Inc., Winooski, USA).

Hemagglutination inhibition (HI) assay. The HI titer was determined as previously described26. Briefly, receptor-destroying enzyme (RDE)-treated sera were serially diluted (twofold) in v-bottom 96-well microtiter plates and 4 hemagglutination units (HAU) of A/Vietnam/1203/2004 (H5N1) (clade 1) or A/Chicken/ Henan/12/2004 (H5N1) (clade 8) whole inactivated viruses were added. Then, 1% (v/v) chicken red blood cells (RBCs) were added and incubated for 30 min at room temperature. HI titer was determined based on the reciprocal value of the last dilution of the sera that completely inhibited hemagglutination of the chicken RBCs. A negative HI titer was defined as less than 10.
H5N1 viruses challenge. Two weeks after the final vaccination, chickens (n = 16 per group) (Fig. 1) were intranasally infected with 25 μL of 10^4 50% egg infective dose (EID_{50}) of A/Vietnam/1203/2004 (H5N1) (clade 1) or A/chicken/Henan/12/2004 (H5N1) (clade 8). The challenged chickens were monitored daily for 14 days to observe changes in body weight and survival rate. Lungs (n = 3 chickens/group) were isolated at day 3 post-challenge to determine viral titers.

Statistical analysis. All data were represented as the mean ± standard deviation (SD). To determine the statistical significance, Kaplan–Meier survival analysis was performed using GraphPad Prism. Two tailed Student’s t test and one-way analysis of variance (ANOVA) were used to determine differences between groups. Values of P < 0.05 were considered statistically significant.

References

1. Tollis, M. & Di Trani, L. Recent developments in avian influenza research: Epidemiology and immunoprophylaxis. Vet. J. 164, 202–215 (2002).
2. Donis, R. O. Antigenic analyses of highly pathogenic avian influenza a virus. Curr. Top. Microbiol. Immunol. 385, 403–440 (2014).
3. Swayne, D. E. Impact of vaccines and vaccination on global control of avian influenza. Avian Dis. 56, 818–828 (2012).
4. Liu, S. et al. Control of avian influenza in China: Strategies and lessons. Transbound Emerg. Dis. 67, 1463–1471 (2020).
5. Swayne, D. E., Pavade, G., Hamilton, K., Vallat, B. & Miyagishima, K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. Rev. Sci. Tech. 30, 839–870 (2011).
6. Jang, H. et al. Efficacy and synergy of live-attenuated and inactivated influenza vaccines in young chickens. PLoS ONE 13, e0195285 (2018).
7. Zhou, E. et al. High-yield production of a stable Vero cell-based vaccine candidate against the highly pathogenic avian influenza virus H5N1. Biochem. Biophys. Res. Commun. 421, 850–854 (2012).
8. Xie, Q. M. et al. Preparation and immune activity analysis of H5N1 subtype avian influenza virus recombinant protein-based vaccine. Poult. Sci. 88, 1608–1615 (2019).
9. Kalemb, B. M. et al. Response to a DNA vaccine against the H5N1 virus depending on the chicken line and number of doses. Virol. J. 17, 66 (2020).
10. Kapczynski, D. R. et al. Vaccination with virus-like particles containing H5 antigens from three H5N1 clades protects chickens from H5N1 and H5N8 influenza viruses. Vaccine 34, 1575–1581 (2016).
11. Lei, H. et al. Protective immunity against influenza H5N1 virus challenge in chickens by oral administration of recombinant Lactococcus lactis expressing neuraminidase. BMC Vet. Res. 11, 85 (2015).
12. Stachura, A. et al. A prime/boost vaccination with HA DNA and Pichia-produced HA protein elicits a strong humoral response in chickens against H5N1. Virus Res. 232, 41–47 (2017).
13. Kim, S. H. & Samal, S. K. Innovation in Newcastle disease virus vectored avian influenza vaccines. Viruses 11, 300 (2019).
14. Suarez, D. L. & Pantin-Jackwood, M. J. Recombinant viral-vectored vaccines for the control of avian influenza in poultry. Vet. Microbiol. 206, 144–151 (2017).
15. Lei, H., Jin, S., Karlsson, E., Schultz-Cherry, S. & Ye, K. Yeast surface-displayed H5N1 avian influenza vaccines. J. Immunol. Res. 2016, 4131324 (2016).
16. Kumar, R. & Kumar, P. Yeast-based vaccines: New perspective in vaccine development and application. FEMS Yeast Res. 19, foa007 (2019).
17. Holmgren, J. & Czerkinsky, C. Mucosal immunity and vaccines. Nat. Med. 11, S45–S53 (2005).
18. Miquel-Clopés, A., Bentley, E. G., Stewart, J. P. & Carding, S. R. Mucosal vaccines and technology. Clin. Exp. Immunol. 196, 205–214 (2019).
19. Dangarembizi, R. et al. Brewier’s yeast is a potent inducer of fever, sickness behavior and inflammation within the brain. Brain Behav. Immun. 68, 211–223 (2018).
20. Lei, H., Xie, B., Gao, T., Cen, Q. & Ren, Y. Yeast display platform technology to prepare oral vaccine against lethal H7N9 virus challenge in mice. Microb. Cell Fact. 19, 53 (2020).
21. Elizaveta, B. & Alexandra, G. Live poultry vaccines against highly pathogenic avian influenza viruses. MIR. J. 5, 22–28 (2018).
22. Doherty, P. C., Turner, S. J., Webby, R. G. & Thomas, P. G. Influenza and the challenge for immunology. Nat. Immunol. 7, 449–455 (2006).
23. Prabakaran, M. et al. Protective immunity against influenza H5N1 virus challenge in mice by intranasal co-administration of baculovirus surface-displayed HA and recombinant CTB as an adjuvant. Virology 380, 412–420 (2008).
24. Wells, J. M. & Mercenier, A. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat. Rev. Microbiol. 6, 349–362 (2008).
25. Neutra, M. R. & Kozlowski, P. A. Mucosal vaccines: The promise and the challenge. Nat. Rev. Immunol. 6, 148–158 (2006).
26. Thomas, P. G. et al. An unexpected antibody response to an engineered influenza virus modifies CD8+ T cell responses. Proc. Natl. Acad. Sci U.S.A. 103, 2764–2769 (2006).
27. de Jong, J. C. et al. Haemagglutination-inhibiting antibody to influenza virus. Dev. Biol. 115, 63–73 (2003).

Author contributions

H.L. conceived and designed the study. X.L., S.L. and Y.R. contributed to animal study and data interpretation. All contributed to data analysis and results interpretation. H.L. wrote the manuscript and produced all figures. All authors reviewed and approved the manuscript.

Funding

Funding was provided by National Natural Science Foundation of China grant number 31360225, Sichuan Science and Technology Program grant number 2019YFN0134 and the Fundamental Research Funds for the Central Universities grant number 2682020ZT87.
Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-88413-2.

Correspondence and requests for materials should be addressed to H.L.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021