Cross-protective immunity of the hemagglutinin stalk domain presented on the surface of Lactococcus lactis against divergent influenza viruses in mice

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Research

Keywords: L.lactis/pNZ8150-pgsA-HAs, Cross-protection, Influenza A viruses

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Cross-protective immunity of the hemagglutinin stalk domain presented on the surface of *Lactococcus lactis* against divergent influenza viruses in mice

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Abstract

Background

Most of the current approaches to influenza vaccine design focus on antibodies against influenza hemagglutinin (HA). However, these influenza vaccines typically provide strain-specific protection against mostly homologous subtypes. There is an urgent need to develop a universal vaccine that confers cross-protection against influenza viruses. Of note, the HA stalk domain (HAs) is a new target for such an influenza vaccine.

Results

Recombinant *L.lactis*/pNZ8150-pgsA-HAs constructed in which pgsA was used as an anchor protein and investigated the immunogenicity of HAs, in the mouse model by oral administration without the use of a mucosal adjuvant. Mice were orally vaccinated with *L.lactis*/pNZ8150-pgsA-HAs, and then produced significant humoral and mucosal immune responses. Importantly, *L.lactis*/pNZ8150-pgsA-HAs provided significant cross-protection against H5N1, H3N2 or H1N1 virus infection.

Conclusions

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Our data support the hypothesis that HAs presented on the surface of *L. lactis* can provide cross-protective immunity against influenza A viruses. Taken together, these findings suggest that *L.lactis*/pNZ8150-pgsA-HAs can be considered an alternative approach to developing a novel universal vaccine during an influenza A pandemic.

Keywords: *L.lactis*/pNZ8150-pgsA-HAs, Cross-protection, Influenza A viruses.

Background

Influenza A viruses cause a highly infectious respiratory disease that remains a public health problem worldwide [1]. Humans can also be infected with influenza viruses that are routinely circulating in animals, such as avian influenza virus subtypes A (H5N1) and swine influenza virus subtypes A (H3N2 and H1N1). Vaccination is considered the most effective way to prevent and control influenza A viruses, although influenza A vaccines must be reformulated annually to match well with the predicated circulating strains [2, 3]. Additionally, most licensed inactivated influenza A vaccines focus on the globular head domain of the major surface glycoprotein of hemagglutinin (HA) of the virus. These vaccines mainly induce strain-specific neutralizing antibodies, which target the highly variable regions of the globular head in the HA1 subunit. Therefore, they provide a limited breadth of protection [4, 5]. In fact, antibodies against HA are the major metric by which immunity to influenza is measured, and they fall into two basic categories: globular- and stalk-directed antibodies [6]. Compared with the high mutability of the globular head region, the HA stalk domain (HAs) is less susceptible to mutations and is relatively conserved across the divergent influenza subtypes [7]. Recent studies have shown that neutralizing antibodies against the HAs can be broadly protective in passive transfer-challenge in the mouse or ferret model [8-12. Thus, the HAs is an alternative target for a novel universal influenza vaccine because antibodies against the HAs are capable of neutralizing diverse influenza A viruses [13].

Lactococcus lactis (*L. lactis*) has been engineered to express heterologous proteins [14]. Our previous studies have indicated that *L. lactis*, expressing the HA, HA1, neuraminidase or nucleoprotein of avian influenza H5N1 virus, is a safe and effective vaccine candidate against

H5N1 virus infection in the mouse or chicken model with the use of a mucosal adjuvant or enteric capsule [15-18]. However, little is known regarding whether the HAs, presented on the surface of *L.lactis* in the absence of a mucosal adjuvant, can provide cross-protective immunity against divergent influenza A viruses.

Thus, to investigate the immunogenicity of the HAs presented on the surface of *L. lactis,* recombinant *L.lactis/*pNZ8150-pgsA-HAs was constructed in which pgsA was used as an anchor protein. Mice were administered orally recombinant *L.lactis/*pNZ8150-pgsA-HAs, which elicited a robust humoral and mucosal immune responses against influenza A viruses. Most importantly, unadjuvanted *L.lactis/*pNZ8150-pgsA-HAs could provide cross-protective immunity against lethal challenge with homologous and heterologous influenza A viruses. These data suggest that recombinant *L.lactis/*pNZ8150-pgsA-HAs is expected to serve as an alternative approach for a truly protective universal influenza vaccine against divergent influenza A viruses.

Methods

Construction of recombinant *L.lactis/*pNZ8150-pgsA-HAs

The HAs (876 bp) of A/Vietnam/1203/2004(H5N1) that including HA 2 and a part of HA1 (**Fig. 1a**) was PCR-amplified from pcDNA3.1-HA (kindly provided by St. Jude Children's Hospital, Memphis, TN, USA) using the following primers with *Nde* I or *EcoR* I sites underlined (forward primer: 5' CTACATATG TCC ACC ATCATGAAATCCGAACTGGAGTAC 3', reverse primer: 5' CCGGAATTC TTAGTTGCAGATGCGACACTGGA 3'). The resulting *Nde* I/*EcoR* I fragment was cloned

into pNZ8150-pgsA at the C-terminal end, in which pgsA was used as an anchor protein [16], and then electroporated into competent *L. lactis* NZ9000. The positive clone of *L.lactis*/pNZ8150-pgsA-HAs was screened and expressed as described previously [16]. *L.lactis*/pNZ8150-pgsA was used as a negative control for subsequent analysis.

Western blot analysis

The expression level of HAs displayed on recombinant *L. lactis* surface was determined by western blot analysis as described previously [16]. Briefly, 10^6 cells of *L.lactis*/pNZ8150-pgsA-HAs pellets were mixed with 60 μL of 6 × loading buffer and boiled for 10 minutes, then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, California, USA). The membrane was incubated with a 1:500 monoclonal mouse anti-HA antibody (kindly provided by NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA, USA), after blocking with 5% skim milk at room temperature for 2 h. After it was incubated overnight at 4 ℃, the membrane was incubated with affinity-purified horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich Corporation, St. Louis, MO, USA). The membrane was reacted with the West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and imaged using the Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunofluorescence assay (IFA)

The HAs protein displayed on *L. lactis* surface was analyzed by an immunofluorescence assay (IFA) (Leica, Wetzlar, Germany). Briefly, 10^6 *L.lactis*/pNZ8150-pgsA-Has cells were washed three times with sterile phosphate-buffered saline (PBS), incubated with monoclonal mouse

anti-HA antibody (kindly provided by NIH Biodefense and Emerging Infections Research Resources Repository, Manassas, VA, USA) at 4 ℃ for 1 h, followed by a FITC-conjugated goat anti-mouse IgG at 4 ℃ for 30 min, and re-suspended with 100 μl of sterile PBS. Finally, *L.lactis*/pNZ8150-pgsA-HAs cells were used for the immunofluorescence assay (IFA). *L.lactis*/pNZ8150-pgsA cells were used as negative controls.

Flow cytometric analysis

Flow cytometry is very similar to IFA, as described above. 500 μL of *L.lactis*/pNZ8150-pgsA-HAs were analyzed by flow cytometric analysis (BD FacsCalibur, BD Bioscience, San Jose, CA, USA).

Animals, vaccine, immunization and sample collection

Specific-pathogen-free (SPF) six-week-old female BALB/c mice were used (SLC Company, Shanghai, China) in this study and housed in cages ventilated under negative pressure with HEPA-filtered air.

The concentration of recombinant *L.lactis*/pNZ8150-pgsA-HAs cells was adjusted to 10^{12} colony forming unit (CFU)/ml with sterile PBS. The mice (n=15 per group) were vaccinated orally with 10^{12} CFU of *L.lactis*/pNZ8008-Spax-HA2 at days 0, 1, 2, and 3 for prime immunization and boosted at day 17, 18, 19, and 20. PBS and *L.lactis*/pNZ8150-pgsA cells were used as controls.

At day 15 and day 34 after the initial immunization, blood samples, intestine and upper respiratory washes were collected, respectively.

Two weeks after the last immunization, all vaccinated mice were transferred into an animal BSL-3 containment facility and challenged intranasally with 20 μl of 10^4 EID_{50} of lethal dose

of A/Vietnam/1203/2004(H5N1), A/Beijing/47/1992 (H3N2) or A/California*/04/2009(*H1N1*)* virus. The mice were monitored for 14 days and body weight loss and survival rate at post challenge were calculated.

Additionally, lungs from vaccinated mice were harvested for the detection of virus shedding at day 3 post-infection. Tissue samples were homogenized and processed as described previously [15]. The virus titer in each sample was calculated by the Reed and Muench method [19] and expressed as 50% tissue culture infective dose ($TCID_{50}$).

All animal immunizations were carried out at biosafety level 2 (BSL-2). Virus challenge experiments must be strictly performed under the enhanced bio-safety level-3 laboratory (BSL-3) containment facilities complying with the Guidelines for Use and Care of Experimental Animals and were approved by the Animal Committee of the Institute of Nanchang University.

Enzyme–Linked Immunosorbent Assay (ELISA)

Antibody responses of serum IgG and IgA in the intestinal washes and upper respiratory washes were determined by (ELISA) using recombinant HA protein (2 μg/ml) as a coating antigen, as described previously [16]. Optical density (OD) was measured at 405 nm using an ELISA plate reader. The IgG or IgA titer was determined to be the lowest dilution with an OD greater than the mean OD of naïve controls plus 2 standard deviations.

Statistical Analysis

A two-tailed Student's t-test and one-way ANOVA were used for all statistical analyses. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

HAs protein expressed on the surface *L.lactis*

The HAs gene of A/Vietnam/1203/2004 (H5N1) (**Fig. 1a**) was cloned into the C-terminal end of pNZ8150-pgsA via a GS linker (**Fig. 1b**). The level of HAs protein expressed on *L. lactis* was determined by western blot analysis. A highly specific band (approximately 100 kDa) was detected for the pgsA-HAs fusion protein in the *L.lactis*/pNZ8150-pgsA-HAs cells (**Fig. 1c, Lane 1**). In contrast, there was no band detected in the *L.lactis*/pNZ8150-pgsA cells (**Fig. 1c, Lane 2**).

To confirm that HAs protein was expressed on the surface of *L. lactis*, *L.lactis*/pNZ8150-pgsA and *L.lactis*/pNZ8150-pgsA-HAs cells were examined by IFA and flow cytometric analysis, respectively. There were no positive signals in the *L.lactis*/pNZ8150-pgsA cells(**Fig. 1 d** and **e**, **left side**). By contrast, *L.lactis*/pNZ8150-pgsA-HAs consistently exhibited specific fluorescent signals, indicating that HAs was stably expressed on the surface of *L. lactis* (**Fig. 1 d** and **e**, **right side**).

Antibody responses detected by ELISA

HA-specific antibody responses were measured by ELISA. As shown in **Fig. 2a**, there was no significant serum IgG detected in the PBS, *L.lactis*/pNZ8150-pgsA or *L.lactis*/pNZ8150-pgsA-HAs group at the prime immunization. A highly significant increase was observed in the *L.lactis*/pNZ8150-pgsA-HAs group at the boost immunization, whereas there were still no significant changes in the PBS or *L.lactis*/pNZ8150-pgsA group.

Mucosal IgA antibodies were also detected in the intestine and upper respiratory washes (**Fig. 2b** and **c**), respectively. There were no detectable IgA antibodies in the PBS, *L.lactis*/pNZ8150-

pgsA or *L.lactis*/pNZ8150-pgsA-HAs group at the prime and boost immunization. Only *L.lactis*/pNZ8150-pgsA-HAs could induce a higher level of IgA antibodies after the boost immunization.

Collectively, these results demonstrated that mice administrated orally with *L.lactis*/pNZ8150-pgsA-HAs after the prime-boost immunization could produce a higher level of HA-specific IgG and IgA antibodies responses.

Cross-protection against virus challenge

Lastly, to assess the cross protective efficacy of *L.lactis*/pNZ8150-pgsA-HAs, all vaccinated mice were challenged with 20 μl of 10^4 EID_{50} of A/Vietnam/1203/2004(H5N1), A/Beijing/47/1992 (H3N2) or A/California/04/2009(H1N1) and monitored for 14 days. As shown in **Fig. 3**, the control groups that received PBS or *L.lactis*/pNZ8150-pgsA showed significant body weight loss and a higher virus shedding in the lung, and died within 8 days after the lethal dose of virus challenge (**Fig.3 a, b** and **c**). In contrast, mice vaccinated orally with *L.lactis*/pNZ8150-pgsA-HAs experienced only mild weight loss and recovered by 14 days (**Fig.3 a, b** and **c**). Further, relatively lower lung virus titers were detected at 3 days post-challenge in the *L.lactis*/pNZ8150-pgsA-HAs group (**Fig.3 d, e** and **f**). Importantly, *L.lactis*/pNZ8150-pgsA-HAs provided 100%, 80% an 80% against A/Vietnam/1203/2004(H5N1), A/Beijing/47/1992 (H3N2) and A/California/04/2009(H1N1), respectively (**Fig.3 g, h** and **i**).

Discussion

Due to continuous antigen changes, the influenza A virus is considered that has the potential to

be the next pandemic [2]. Most of the currently available vaccines against influenza only provide stain-specific protection and require annual reformulation as the influenza virus can escape vaccine-induced humoral immunity [19]. Therefore, there is an urgent need to develop a universal influenza vaccine that relies on the utilization of highly conserved antigenic targets such as M2 and NP [20, 21]. However, these conserved antigen epitopes are usually less exposed to the host immune system, and as such, are naturally weakly immunogenic [22]. Influenza A HA consists of two subunits: HA1, which forms a globular head and HA2, which forms a stalk domain [10]. Antibodies against the HAs can elicit broad neutralizing activity and provide cross-protection against heterologous influenza viruses [8, 23, 24]. Based on these previous findings, the HAs is an attractive target for developing a novel universal influenza vaccine.

It has been hypothesized that the HAs presented on the surface of *L.lactis* may provide cross-protective immunity against divergent influenza A viruses. In this study, HAs, including HA 2 and a part of HA1 (**Fig. 1a**), was displayed on the surface of *L.lactis, L.lactis*/pNZ8150-pgsA-HAs showed a specific binding profile that could be labelled directly with anti-HA antibody and FITC-conjugated goat anti-mouse IgG. It was then tested by immunofluorescence assay and flow cytometric analysis (**Fig. 1d** and **e**). The binding profiles observed positively correlated with the display efficacy of HAs, demonstrating that HAs presented on the surface of *L. lactis* has reactive activity in vitro, which may contribute to immunogenicity in vivo.

In the mouse model, the contributions of HA-specific antibodies for broad-spectrum protection against divergent influenza A viruses were emphasized. Oral vaccination with *L.lactis*/pNZ8150-pgsA-HAs could elicit higher humoral and mucosal immune responses

compared with *L.lactis*/pNZ8150-pgsA (**Fig.2a, b** and **c**). Further, virus challenge is regarded as the gold-standard for assessing the efficacy of an influenza vaccine. Mice administered orally with *L.lactis*/pNZ8150-pgsA-HAs were protected completely (100%) from homologous A/Vietnam/1203/2004(H5N1) virus challenge, as well as 80% against heterologous H3N2 or H1N1 virus infection (**Fig. 3g**, **h** and **i**), and showed lower degree of virus shedding in the lung (**Fig. 3d**, **e** and **f**). These findings provide reliable evidence that *L.lactis*/pNZ8150-pgsA-HAs is an effective vaccine candidate to induce cross protective immunity against divergent influenza A viruses.

It has been demonstrated previously that adjuvanted *L.lactis*/pNZ8110-pgsA-HA1, in which pgsA was used as an anchor protein, provided immune protection against homologous H5N1 virus in the mouse model [16]. In this study, pgsA was designed to display HAs and also showed stable characterization on the *L. lactis* surface. Based on these findings, presenting HAs on the surface of *L. lactis* may provide an alternative approach to novel universal influenza vaccine development.

Conclusions

The HAs represents a new research target for a novel universal influenza vaccine. Notably, the HAs presented on the surface of *L. lactis* has the potential for inducing cross-protective immunity against divergent influenza A viruses. Thus, this study strongly demonstrates that the discovery of *L.lactis*/pNZ8150-pgsA-HAs may allow for an alternative design of influenza vaccines that would afford broad coverage.

Authors' contributions

HL, TG and QC contributed to study design and data interpretation. HL wrote the manuscript and produced all figures. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All animal studies complied with the Guidelines for Use and Care of Experimental Animals and were approved by the Animal Committee of the Institute of Nanchang University consent to participate.

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Fig. 1. Expression of HAs presented on the *L. lactis* surface. **(a)** Schematic description of the full length of HA from the H5N1 strain containing a globular head and stalk domain. SP: Signal peptide; FP: Fusion peptide; TM: Transmembrane domain; CT: Cytoplasmic tail. **(b)** Schematic diagram of pNZ8150-pgsA-Has. A GS linker was inserted between pgsA and HAs to stabilize the HAs protein expression. **(c)** Western blot analysis. Lane 1: *L.lactis*/pNZ8150-pgsA-HAs; Lane 2: *L.lactis*/pNZ8150-pgsA. **(d)** Immunofluorescence microscopy assay. *L.lactis*/pNZ8150-pgsA (left) and *L.lactis*/pNZ8150-pgsA-HAs (right) (magnification: 1, 000×). **(e)** Flow cytometric analysis (positive rate: 45.9%).

Fig. 2. Antibody responses detected by ELISA and HI assay. Sera, intestine and upper respiratory washes were collected from the vaccinated mice at day 15 and day 34 after the initial immunization. **(a)** HA-specific IgG antibody responses in the sera. **(b)** HA-specific IgA antibody responses in the intestine washes. **(c)** HA-specific IgA antibody responses in the upper respiratory washes. The data are represented as the mean ± SD. Asterisk indicates significant difference compared with PBS and *L.lactis*/pNZ8150-pgsA controls ($p < 0.05$).

Fig. 3. Cross-protective efficacy against divergent influenza A viruses. The results are expressed in terms of percent body weight **(a, b and c)**, lung virus titers **(d, e and f)** and percent survival **(g, h andi)**. Two weeks after the last immunization, mice were intranasally infected with 20 μl of 10^4 EID_{50} of lethal dose of A/Vietnam/1203/2004(H5N1), A/Beijing/47/1992

(H3N2) and A/California/04/2009(H1N1) (C, F and I) (n=5 / group). The data for lung virus titers are represented as the mean ± SD. Asterisk indicates significant difference compared with PBS and *L.lactis*/pNZ8150-pgsA controls ($p < 0.05$).

Figures

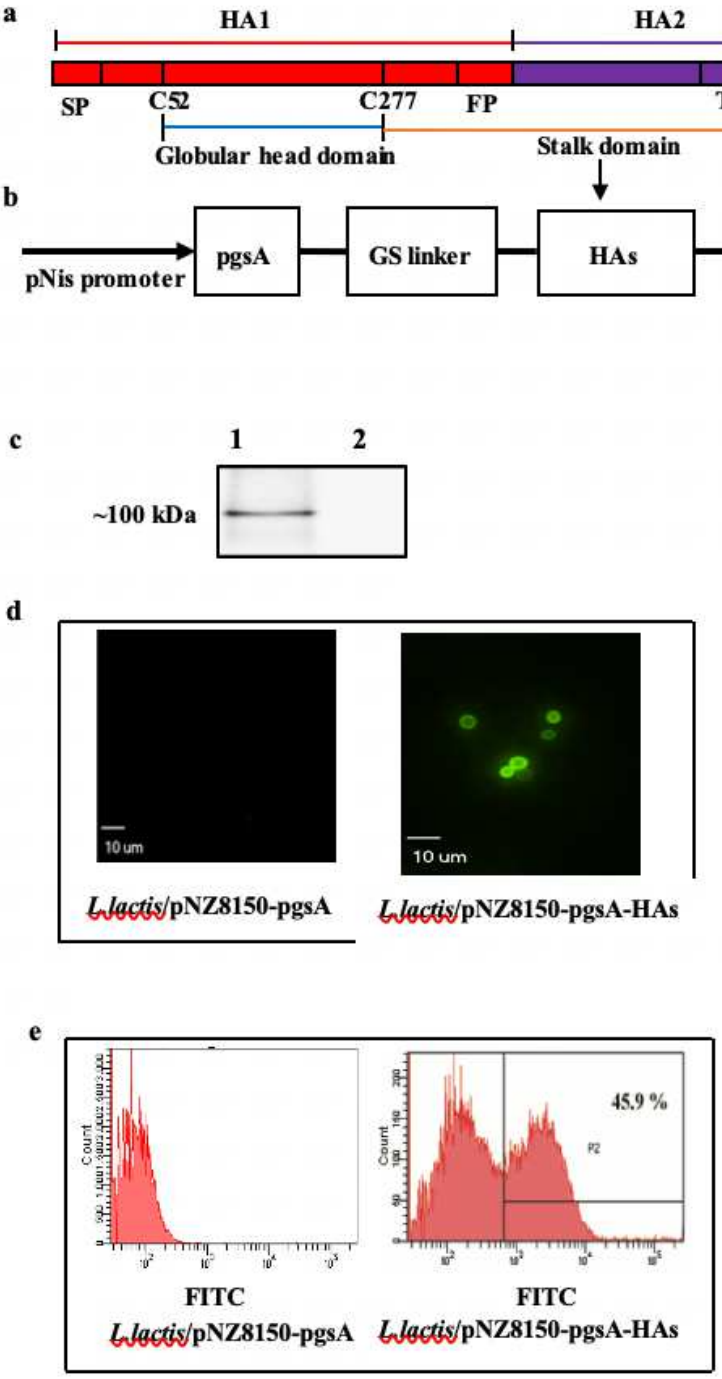

Figure 1

Expression of HAs presented on the L. lactis surface. (a) Schematic description of the full length of HA from the H5N1 strain containing a globular head and stalk domain. SP: Signal peptide; FP: Fusion peptide; TM: Transmembrane domain; CT: Cytoplasmic tail. (b) Schematic diagram of pNZ8150-pgsA-

Has. A GS linker was inserted between pgsA and HAs to stabilize the HAs protein expression. (c) Western blot analysis. Lane 1: L.lactis/pNZ8150- pgsA-HAs; Lane 2: L.lactis/pNZ8150-pgsA. (d) Immunofluorescence microscopy assay. L.lactis/pNZ8150-pgsA (left) and L.lactis/pNZ8150-pgsA-HAs (right) (magnification: 1, 000×). (e) Flow cytometric analysis (positive rate: 45.9%).

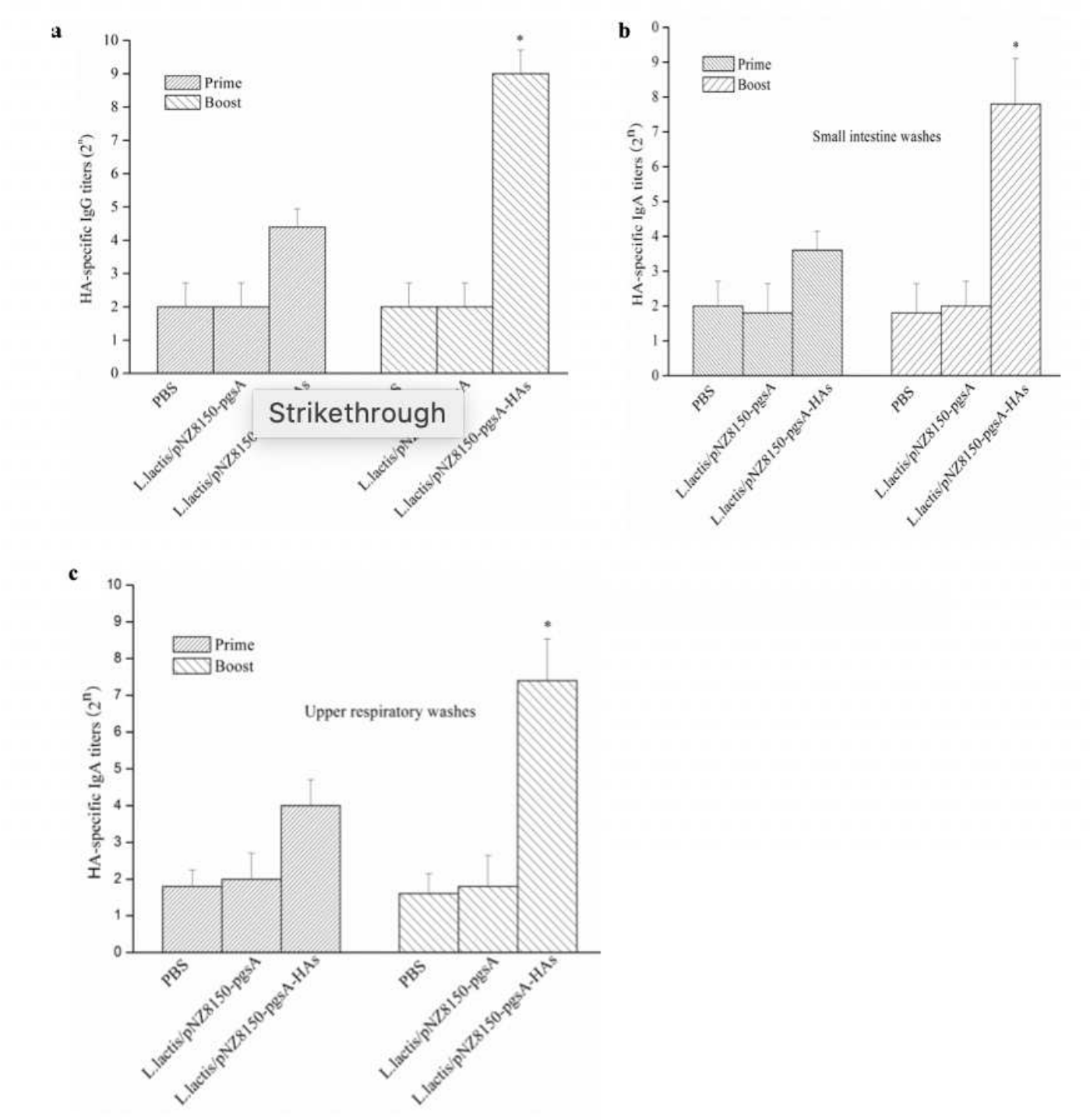

Figure 2

Antibody responses detected by ELISA and HI assay. Sera, intestine and upper respiratory washes were collected from the vaccinated mice at day 15 and day 34 after the initial immunization. (a) HA-specific IgG antibody responses in the sera. (b) HA-specific IgA antibody responses in the intestine washes. (c) HA-specific IgA antibody responses in the upper respiratory washes. The data are represented as the mean ± SD. Asterisk indicates significant difference compared with PBS and L.lactis/pNZ8150-pgsA controls (p □ 0.05).

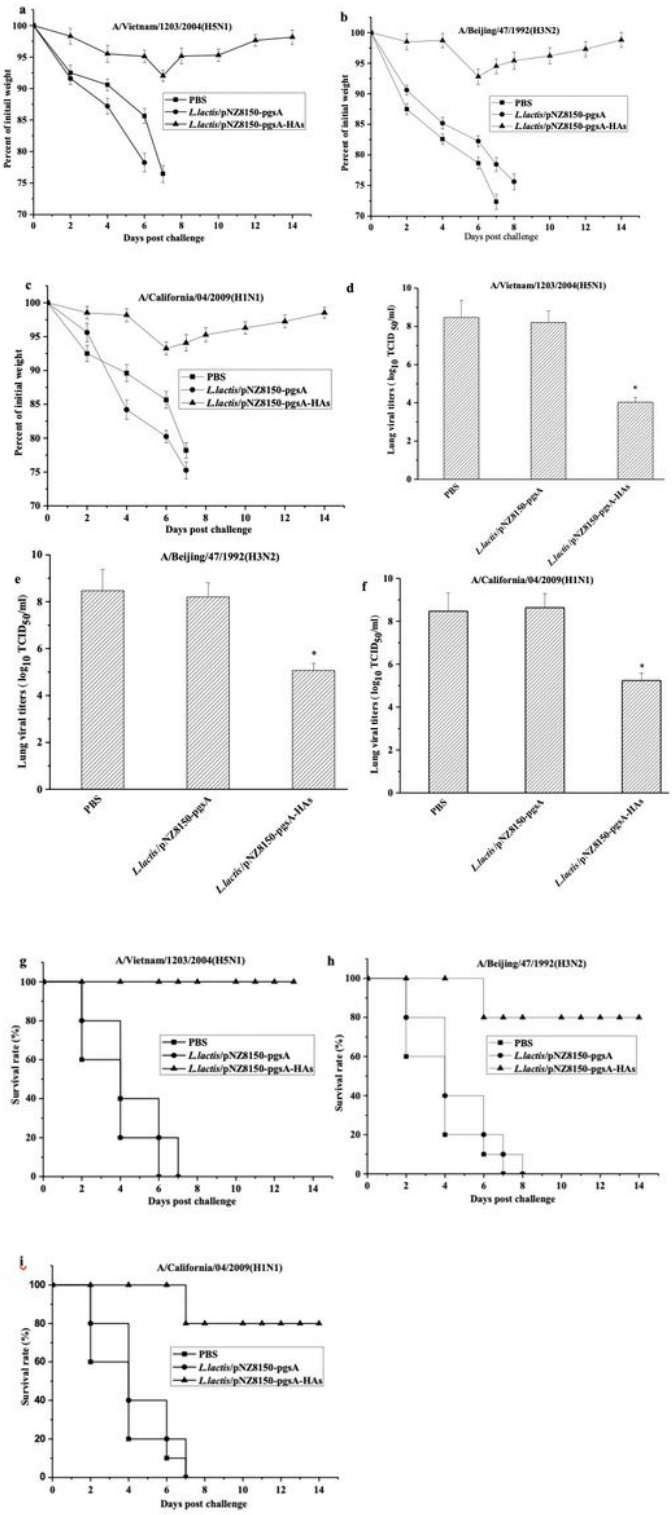

Figure 3

Cross-protective efficacy against divergent influenza A viruses. The results are expressed in terms of percent body weight (a, b and c), lung virus titers (d, e and f) and percent survival (g, h andi). Two weeks after the last immunization, mice were intranasally infected with 20 μl of 104 EID50 of lethal dose of A/Vietnam/1203/2004(H5N1), A/Beijing/47/1992 (H3N2) and A/California/04/2009(H1N1) (C, F and I) (n=5 / group). The data for lung virus titers are represented as the mean ± SD. Asterisk indicates significant difference compared with PBS and L.lactis/pNZ8150-pgsA controls (p 0.05).