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Virology

Immunoprotection against influenza virus H9N2 by the oral administration of recombinant *Lactobacillus plantarum* NC8 expressing hemagglutinin in BALB/c mice ☆

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ABSTRACT

The H9N2 avian influenza virus (AIV) has become increasingly concerning due to its role in severe economic losses in the poultry industry. Transmission of AIV to mammals, including pigs and humans, has accelerated efforts to devise preventive strategies. To develop an effective oral vaccine against H9N2 AIV, a recombinant *Lactobacillus plantarum* NC8 strain expressing the hemagglutinin (HA) gene of H9N2 AIV was constructed in this study. Mice were orally immunized with the recombinant NC8-pSIP409-HA strain, and sIgA, IgG and HI antibodies were produced by the NC8-pSIP409-HA strain, which also induced CD8^{+} T cell immune responses. Most importantly, oral administration produced complete protection against challenge with mouse-adapted H9N2 virus. These results indicate that the recombinant NC8-pSIP409-HA was more effective at inducing the mucosal, humoral and cellular immune responses. Therefore, *L. plantarum* NC8-pSIP409-HA could become a promising oral vaccine candidate against H9N2 AIV.

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Introduction

Low pathogenic avian influenza (LPAI) H9N2 viruses have circulated widely around the world and have been isolated from terrestrial poultry worldwide (Chen et al., 2003; Rahman et al., 2011; Xu et al., 2007b). Since the outbreak of LPAI H9N2 in 1996 (Lee et al., 2000), the virus has infected a high proportion of chickens and other land-based birds such as pigeons, pheasants, quails, chukkas and turkeys (Coman et al., 2013; Guan et al., 2000). It has received considerable attention because LPAI H9N2-infected chickens are vulnerable to secondary infection with pathogenic microbes, which may cause serious commercial economic losses in the poultry industry (Rahman et al., 2011). More seriously, chickens infected with LPAI H9N2 viruses not only can serve as a reservoir host but also can transmit the virus to mammals such as cats, dogs, pigs and people (Alexander, 2000; Blair et al., 2013; Choi et al., 2004; Coman et al., 2013; Peiris et al., 2001; Webster et al., 1992). Moreover, H9N2 viruses were also found in patients with influenza-like symptoms in southeast China and Hong Kong SAR (Peiris et al., 1999). In addition, viruses isolated from humans, such as A/HongKong/1073/1999 and A/Hong Kong/33982/2009, had the highest risk potential. However, the A/swine/Hong Kong/9A-1/1998 and A/chicken/Hong Kong/G9/1997 viruses also displayed several features that suggested a profile that was adapted to human infection and transmission (Group, 2013). Importantly, the six internal genes of novel H7N9 viruses were derived from LPAI H9N2 (Gao et al., 2013; Liu et al., 2013). LPAI H9N2 viruses comprise a genetically diverse population that infects wild species and mammals; they contributed the internal gene segments to the A/H7N9 viruses associated with lethal human infections, and different H9N2 strains have different ecological profiles and risks.

These cases indicate that there is an urgent need to control or prevent the occurrence of LPAI H9N2 disease.

Although vaccination will not solve all of these problems, it is one of the most promising control measures for the LPAI H9N2 to date (Rahman et al., 2011). Side effects of the commercialized vaccines (attenuated and inactivated vaccines) are not uncommon. For instance, attenuated immunization is an effective antiviral approach; however, there is a risk of spreading the virus and reacquiring its toxicity (Perelberg et al., 2005). Compared to the attenuated vaccines, inactivated vaccines exhibit increased safety and reduced risk of spreading the disease (Li et al., 2012). Nevertheless, the low immunological protection, instability and the need for repeated immunization measures make these vaccines less desirable. In addition, new vaccine preparations, including various DNA vaccines (Wei et al., 2008), subunit vaccines (Chen et al., 2008) and recombinant adenovirus vaccines (Gao et al., 2006) have been examined, but they all require injections, which would be extremely difficult to perform on wild birds or farm animals. Moreover, the intense stress caused by injection could hamper animal growth (Lee et al., 2006; Wang et al., 2006). Based on these factors, many investigators are pursuing more safe, economical, convenient and efficacious avenues for constructing new vaccine candidates to control LPAI H9N2 disease.

Compared with other new vaccines, oral vaccines cause less stress among animals and have been receiving growing interest due to their advantages over the conventional parenteral vaccines (Wang et al., 2012). The greatest advantage is that these vaccines can trigger mucosal and systemic immune responses against foreign microbial infection via activated dendritic cells (DCs) (Mohamadzadeh et al., 2009; Sim et al., 2008), which migrating to the mesenteric lymph nodes (MLNs) and presenting processed antigens to T and B lymph cells. *Lactobacillus* is a genus of gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria (Wang et al., 2012). These bacteria are the commonly members of the gut commensal community and are among the earliest inhabitants of the gastrointestinal tract without causing disease (Von Ossowski et al., 2013). Characteristically, such bacteria have been shown to have a strong propensity to help promote good health as well as alleviate a variety of health problems (Dicks and Botes, 2010; Doron and Gorbach, 2006; Tannock, 2004), and they are therefore generally regarded as safe (GRAS) (Lei et al., 2010). This approach has been reported in the use of recombinant *lactobacilli* to express exogenous antigens for oral administration (Cho et al., 2010; Gilbert et al., 2000; Robinson et al., 1997; Xin et al., 2003), both in terms of the amount of antigen expression and the duration of persistence in the gut (Klijn et al., 1995).

In the present study, we constructed the recombinant NC8-pSIP409-HA based on *Lactobacillus plantarum* NC8 expressing hemagglutinin (HA) of the H9N2 virus. We researched the immunogenicity of the recombinant NC8-pSIP409-HA in BALB/c mice with respect to the induction of hemagglutination inhibition (HI) antibodies as well as HA-specific serum IgG antibodies, HA-specific mucosal IgA antibodies, IFN-γ-producing CD8$^+$ T cells and CD8$^+$ T cells responses. The protective efficacy of the recombinant NC8-pSIP409-HA against a homologous virus challenge was determined by quantification of the H9N2 virus in the lungs of BALB/c mice, and a mouse-adapted H9N2 strain of enhanced pathogenicity was applied to evaluate the efficacy of the recombinant NC8-pSIP409-HA in promoting the percent survival.

Results

HA expression by recombinant L. plantarum NC8

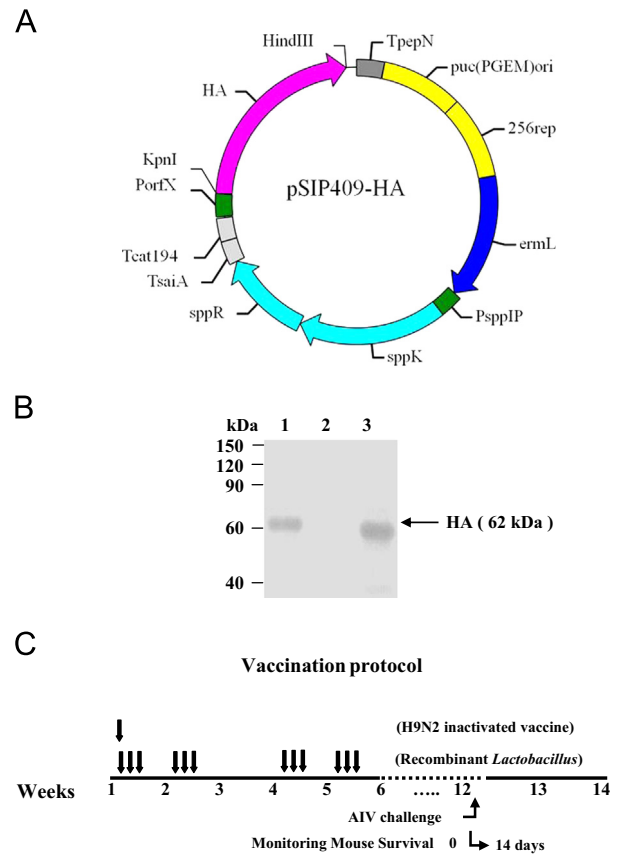pSIP409-HA (Fig. 1A) and pSIP409 were constructed and introduced into *L. plantarum* NC8 by electroporation. The cell

Fig. 1. Plasmids for the expression of recombinant hemagglutinin antigen, western blots of expressed NC8-pSIP409-HA and NC8-pSIP409 proteins and vaccination schedule. (A) The pSIP409-HA plasmid was constructed as described in the text. The plasmid structure of pSIP409-HA (~7.2 kb). HA: hemagglutinin gene; 256rep: replication origin for *Lactobacillus*; ermL: erythromycin-resistance marker; PsppIP and PorfX: inducible promoters; sppK and sppR: histidine protein kinase and response regulator, respectively. (B) The HA protein expressed in NC8-pSIP409-HA was detected by western blotting. M: pre-staining marker, Lane 1: NC8-pSIP409-HA, lane 2: NC8-pSIP409 as the negative control, lane 3: H9N2 virus as positive control. (C) Groups of mice were immunized with NC8-pSIP409-HA, NC8-pSIP409 (10^9 CFU in 200 μl), PBS (200 μl) or H9N2 inactivated vaccine. Immunization via the oral route was repeated thrice weekly. Two weeks later, the groups of mice were boosted twice. Six weeks after the final boost, the mice were challenged i.n. with mouse-adapted H9N2 virus. H9N2 inactivated vaccine was injected intramuscularly on the first day. Survival was then monitored until day 14.

lysates of overnight cultures of NC8-pSIP409-HA or NC8-pSIP409 in basal MRS medium with additional SppIP obtained by centrifugation were subjected to SDS-PAGE and western blotting for examination of the protein expression. An immunoreactive 62-kDa band was detected in the cell lysates (Fig. 1B, lane 1), whereas no band was observed in the negative control lane (Fig. 1B, lane 2). These results showed that HA was expressed by SppIP in *L. plantarum* NC8.

HI titers in the sera of mice immunized with recombinant L. plantarum NC8

The antibody titers in the sera 21, 35 and 49 days after the first immunization were analyzed using an HI assay. As shown in Fig. 2, antibody titers were not detected after immunization with the negative control PBS and NC8-pSIP409, whereas antibody titers increased gradually after immunization with NC8-pSIP409-HA. In addition, the antibody titers produced by the H9N2 inactivated vaccine were tested, but there was no significant difference between NC8-pSIP409-HA and H9N2 inactivated vaccines on day 49.
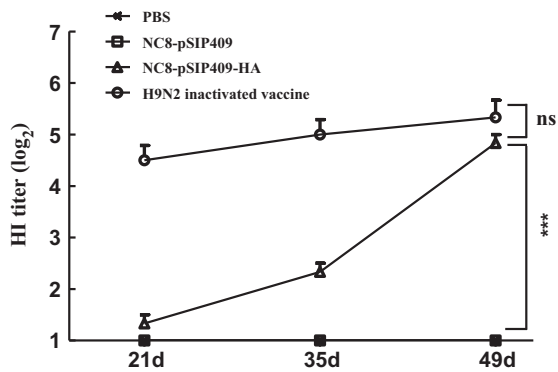
Fig. 2. Antibody levels analyzed using an HI assay. Twenty-one, 35 and 49 days after the mice were immunized with NC8-pSIP409-HA, NC8-pSIP409, PBS and a positive control, HI titers were assessed using an HI assay with the H9N2 AIV and a positive serum antibody. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (***$p < 0.001$). Data are expressed as the mean ± SEM of triplicate experiments. $n = 5$ mice per group.

Ab titers against the HA antigen

To evaluate the immunogenicity of the recombinant NC8-pSIP409-HA, groups of mice were orally immunized, and antibody titers were determined 21, 35 and 49 days after the first immunization. HA-specific IgA levels in the feces and bronchoalveolar lavage fluids (BALF) were detected using ELISA. The group immunized with NC8-pSIP409-HA showed relatively high HA-specific IgA production in the feces and BALF, whereas the NC8-pSIP409 and PBS showed little specific IgA production (Fig. 3A and B). In addition, there was no significant difference between NC8-pSIP409-HA and H9N2 inactivated vaccine. We also detected significant levels of specific IgG in the serum of mice in the NC8-pSIP409-HA group (Fig. 3C); the NC8-pSIP409 and PBS produced no meaningful results regarding antibody (Ab) titers against the HA antigen. In addition, the current AIV inactivated vaccine, administered in a single intramuscular injection, induced higher IgG antibody titers.

The Ab response was also associated with the germinal centers (GC) in the Peyer's patch (PP), MLN and spleens of the immunized mice. PP, MLN cells and splenocytes were removed from the four groups of mice 7 days after the final immunization. PP, MLN and splenocytes were gated on B220$^+$ cells, and staining for the presence of PNA$^+$ and FAS$^+$ B cells was performed. Mice that were orally immunized with NC8-pSIP409-HA developed significant numbers (25.1% of the total isotype-switched B cells) of FAS$^+$ PNA$^+$ B220$^+$ B cells in the PP compared with other groups. In addition, the H9N2 inactivated vaccine also produced significant numbers (28.6% of the total isotype-switched B cells) of FAS$^+$ PNA$^+$ B220$^+$ B cells in PP. However, there is no significant difference between NC8-pSIP409-HA and H9N2 inactivated vaccine. Interestingly, there were also a significant number of FAS$^+$ PNA$^+$ B220$^+$ B cells in the MLN and spleens of mice that were immunized with NC8-pSIP409-HA compared with other groups (Fig. 3D). The results indicated that GC was formed after oral immunization. In addition, BALB/c mice immunized with PBS and NC8-pSIP409 failed to show significant numbers of FAS$^+$ PNA$^+$ B220$^+$ B cells. These results indicate that mice that were immunized with NC8-pSIP409-HA demonstrate an effective B cell response.

Cell-mediated immune responses

Cellular immune responses to NC8-pSIP409-HA were evaluated by measuring IFN-γ secretion in murine MLN cells and splenocytes. Twenty-one days after the final immunization, MLN cells and splenocytes were harvested from each immunization group, and the number of IFN-γ-secreting MLN cells and splenocytes was calculated as the average number of spots in the triplicate stimulant wells. As shown in Fig. 4A and B, a significant number of HA-specific IFN-γ-secreting MLN cells and splenocytes were detected in the immunized group compared with the control group.

We next investigated the role of activated T cells in the immune response. Twenty-one days after the final immunization, MLN cells and splenocytes were harvested from each immunization group, and IFN-γ-producing T cells were analyzed by flow cytometry. The results show that there was a significant increase in the frequency of IFN-γ-producing CD8$^+$ T cells in the MLN and splenocytes of mice immunized with NC8-pSIP409-HA. However, IFN-γ-producing T cells were induced at low levels in mice that were vaccinated with the H9N2 inactivated vaccine (Fig. 4C). In addition, the frequency of IFN-γ-producing CD4$^+$ T cells was not meaningful (data not shown).

T cell proliferation in response to AIV HA antigen restimulation

To test the splenocyte proliferative response upon antigen restimulation, we measured CD8$^+$T cell proliferation in response to AIV HA antigen restimulation. Splenocytes were prepared from mouse spleens 30 days after the booster immunization and were stimulated in vitro with AIV HA. After 3 days of incubation, the CFSE profiles of CD8$^+$ gated T cells were detected using flow cytometry. As shown in Fig. 5, when stimulated with AIV HA, cells isolated from the spleens of mice that were orally administered NC8-pSIP409-HA displayed significant CD8$^+$ T cell proliferation in response to AIV HA antigen restimulation. However, splenocyte proliferation was induced at low levels in mice that were vaccinated with the H9N2 inactivated vaccine. These results showed that immunization with NC8-pSIP409-HA elicited a more efficient and robust splenocyte proliferation response against AIV HA antigen.

Protection against a lethal challenge with mouse-adapted H9N2 influenza virus in mice with the oral administration of NC8-pSIP409-HA

To determine whether NC8-pSIP409-HA provides protection against H9N2 influenza viruses, six weeks after the final immunization, the mice were intranasally challenged with a lethal dose (10 × LD50) of mouse-adapted H9N2 influenza virus and were closely monitored for 14 days for weight loss, mortality and lung virus titer. After the viral challenge, all mice experienced body weight loss (Fig. 6A), but the mice that were immunized with NC8-pSIP409-HA gradually recovered after 7 days, and 100% survival was achieved. In contrast, mice immunized with PBS and NC8-pSIP409, all died within 10 days of the viral challenge (Fig. 6B). The AIV inactivated vaccine, administered in a single intramuscular injection, completely protected the mice from the lethal viral challenge with mouse-adapted H9N2 influenza virus (Fig. 6B). In addition, the residual lung virus titers of the mice that were immunized with NC8-pSIP409-HA and challenged with the H9N2 viruses were significantly lower than the mice in the control groups ($p < 0.001$), as shown in Fig. 6C.

Protection against severe lung pathology in BALB/c mice

To evaluate vaccine efficacy, mice from different groups were killed, and their lungs were examined microscopically. The results indicated that BALB/c mice that were orally vaccinated with NC8-pSIP409-HA and H9N2 inactivated vaccine did not develop the apparent inflammatory changes after challenge with the
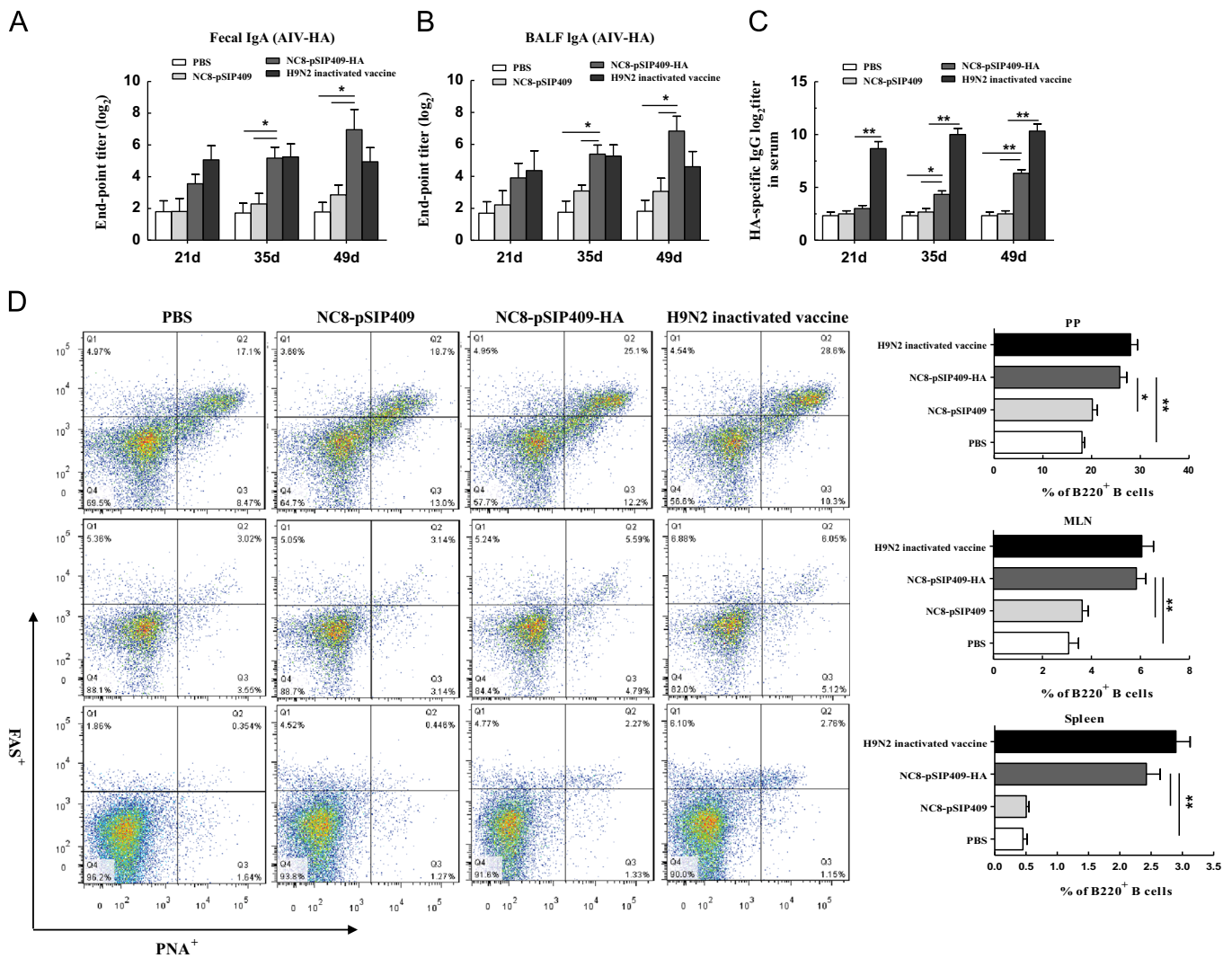
Fig. 3. HA-specific antibody titers detected with ELISA and activated B cells detected with flow cytometry. Measurement of HA-specific immunoglobulin A (IgA) Ab titers in the feces (A) and BALF (B) after immunization. (C) Measurement of HA-specific immunoglobulin G (IgG) Ab titers in the serum after immunization. Immunization conditions are displayed at the bottom. (D) Detection of activated B cells in the germinal centers (GC) of immunized mice using flow cytometry. Representative flow cytometric analyses of GC B cells among $B220^+$ B cells in the Peyer's patches (PP), MLN and spleen 7 days after the final immunization. The % of total cells for the quadrants and the bar graphs refer only to $B220^+$ cells. Values are the percentages of activated GC B cells ($PNA^+ FAS^+$) among the gated $B220^+$ cells. Immunization conditions are shown at the top. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (*$P < 0.05$, **$P < 0.01$). Data are expressed as the mean ± SEM of triplicate experiments. $n=3$ mice per group.

mouse-adapted H9N2 AIV virus, but there was a small number of mononuclear cells detected by HE staining of the H9N2 inactivated vaccine (Fig. 7). On the contrary, the lungs developed alveoli with interstitial edema, a foamy exudate and a large number of mononuclear cells (focal emphysema), and a large number of alveolar structures were destroyed by oral vaccination withNC8-pSIP409 and PBS (Fig. 7). These findings show that prophylactically administered NC8-pSIP409-HA confers protection against the mouse-adapted H9N2 challenge and reduces pulmonary pathology.

Discussion

The H9N2 influenza virus affects humans and can result in an economically important disease in poultry; therefore, many researchers are actively pursuing the development of safe and efficacious oral vaccines, which can be produced economically and conveniently administered. Wild-type H9N2 avian influenza virus A/duck/Xuzhou/07/2003(H9N2) isolated from duck that uninfected mice, it was adapted to growth in mice that can infect mice

and result in dying. In this paper, adaptation of wild-type H9N2 virus was model virus that can evaluate the protective rate of the vaccine in a mouse model. The initial infection with influenza virus mainly occurs at the respiratory tract mucosa, which is the site of defense against virus infection and is a critical step in the infection process (Tamura and Kurata, 2004). Therefore, mucosal administration is the first choice for vaccination as an effective prophylactic method against H9N2 AIV. Mucosal vaccines can induce mucosal immune responses as well as systemic responses, but the current commercialized vaccines can not induce mucosal immune responses. In this regard, most investigators are currently focused on the development of appropriate mucosal vaccines for oral administration.

The use of recombinant vaccines has been documented for influenza viruses previously (Wei et al., 2008; Lee et al., 2006). Indeed, bacterial and viral vectors expressing HA from H9N2 and H5N1 avian influenza have been shown to induce protective immunity. (Pan et al., 2009; Gao et al., 2006). These systems may be more immunogenic or may express stronger immune responses, but most of them were originally pathogenic strains.
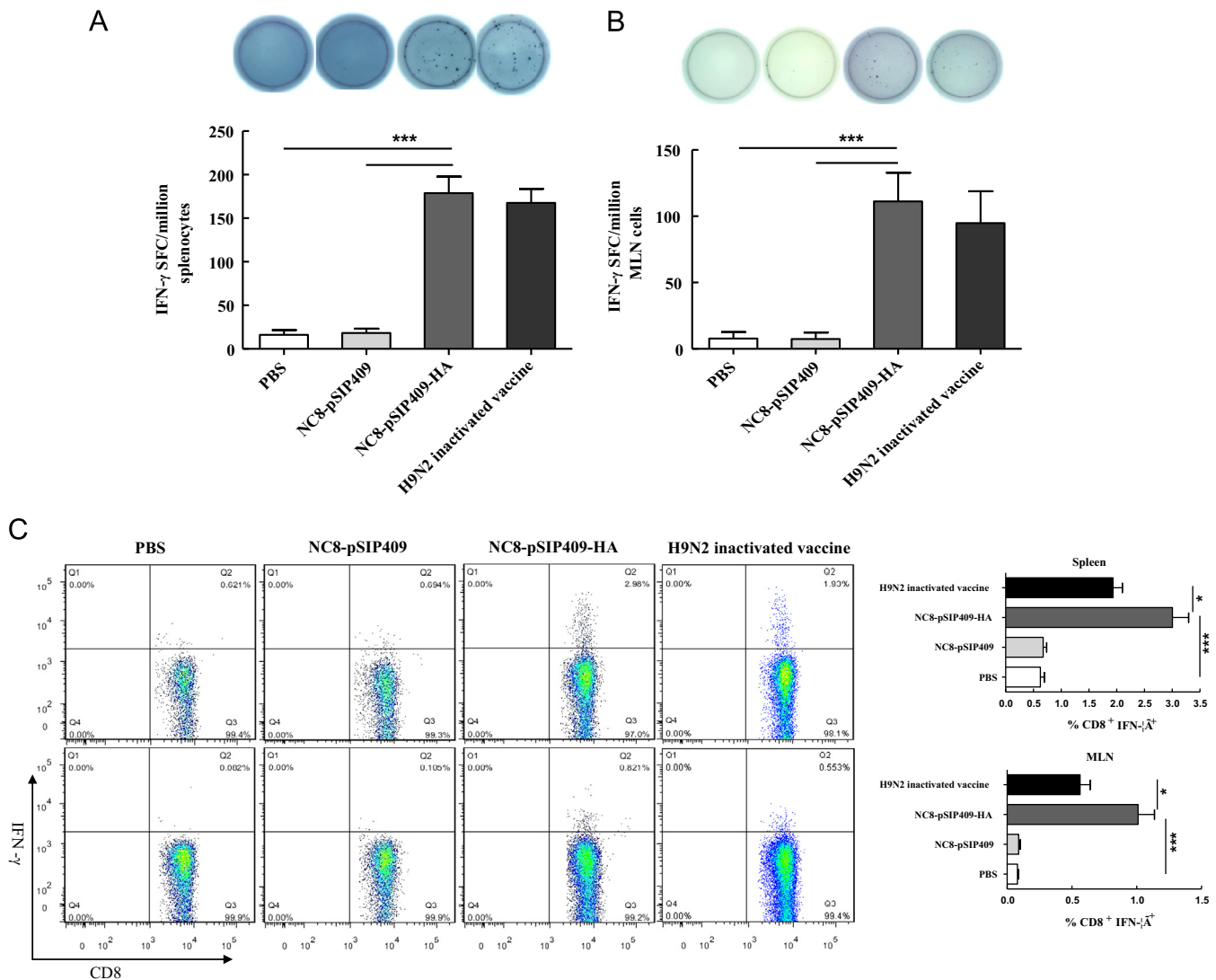
Fig. 4. Cell-mediated immune responses induced by recombinant *Lactobacillus plantarum*. Twenty-one days after vaccination, (A) splenocytes and (B) MLN cells were harvested and analyzed in ELISPOT assays for IFN-γ production following in vitro stimulation with purified HA. (C) Percentages of IFN-γ-producing CD8^{+} T cells in the spleen and MLN 21 days after the final immunization. Splenocytes and MLN cells from immunized mice were stimulated for 12 h with purified HA or a medium control. T cells are labeled with anti-CD3 and identified by their respective surface markers (CD8) and intracellular IFN-γ staining. The immunization conditions are shown at the top. The values in the quadrants are percentages of IFN-γ^{+} CD8^{+} T cells. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (*$P < 0.05$, ***$P < 0.001$). Data are expressed as the mean ± SEM of triplicate experiments. $n = 5$ mice per group.

In contrast, the vectors based on *Lacticacid bacteria* (LAB) are considered safe, are convenient and are widely used as a live vaccine vehicle against various foreign microbes (Peiris et al., 2001; Xu et al., 2007a); however, these may not be as immunogenic for the human immune system. Moreover, many researchers reported that specific or memory B cells reactive to H5N1 and H1N1 HA were indeed present in individuals, strongly suggesting that the vaccine preferentially activates these memory B cells, thereby producing a humoral response with neutralizing activity (Whittle et al., 2014; Li et al., 2012). Further studies of memory B-cell responses to *Lactobacillus*-based vaccines and natural chicken AIV infection are needed.

Grönqvist and his colleagues initiated the development of one-plasmid inducible expression systems based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin P (spp gene cluster), and they constructed prototypes of the modular so-called pSIP expression vectors (Grönqvist et al., 2003). The pSIP-409 vector in this study contains a promoter element derived from the sakacin P structural gene with an engineered *Nco*I site for translational fusion cloning,

which encodes the cognate histidine protein kinase and response regulator that are necessary to activate this promoter upon induction by sakacin P and gusA reporter gene (Sørvig et al., 2005). The target protein expression was completely dependent on the SppIP induction. In addition, the recombinant LAB vector was used to produce large quantities of antigens. Although constitutive expression causes improved immunogenicity, an inducible expression system can relieve the pressure of the exogenous proteins on LAB, compared to a constitutive expression system. LAB may have been able to persist over approximately 6 days, preventing immunotolerance against the target antigen (Xu et al., 2007c). Thus, to mitigate the metabolic stress on the host bacteria, inducible promoters were chosen in *L. plantarum*.

The oral administration of recombinant *L. plantarum* can induce secretory IgA antibodies in the respiratory tract, which is the major target organ of influenza virus infection. Secretory IgA antibodies are considered a major effector in the adaptive immune defense of the respiratory mucosa (Underdown and Schiff, 1986). Although oral administration of *L. plantarum* or recombinant *Lactococcus lactis* protects against influenza virus infection by
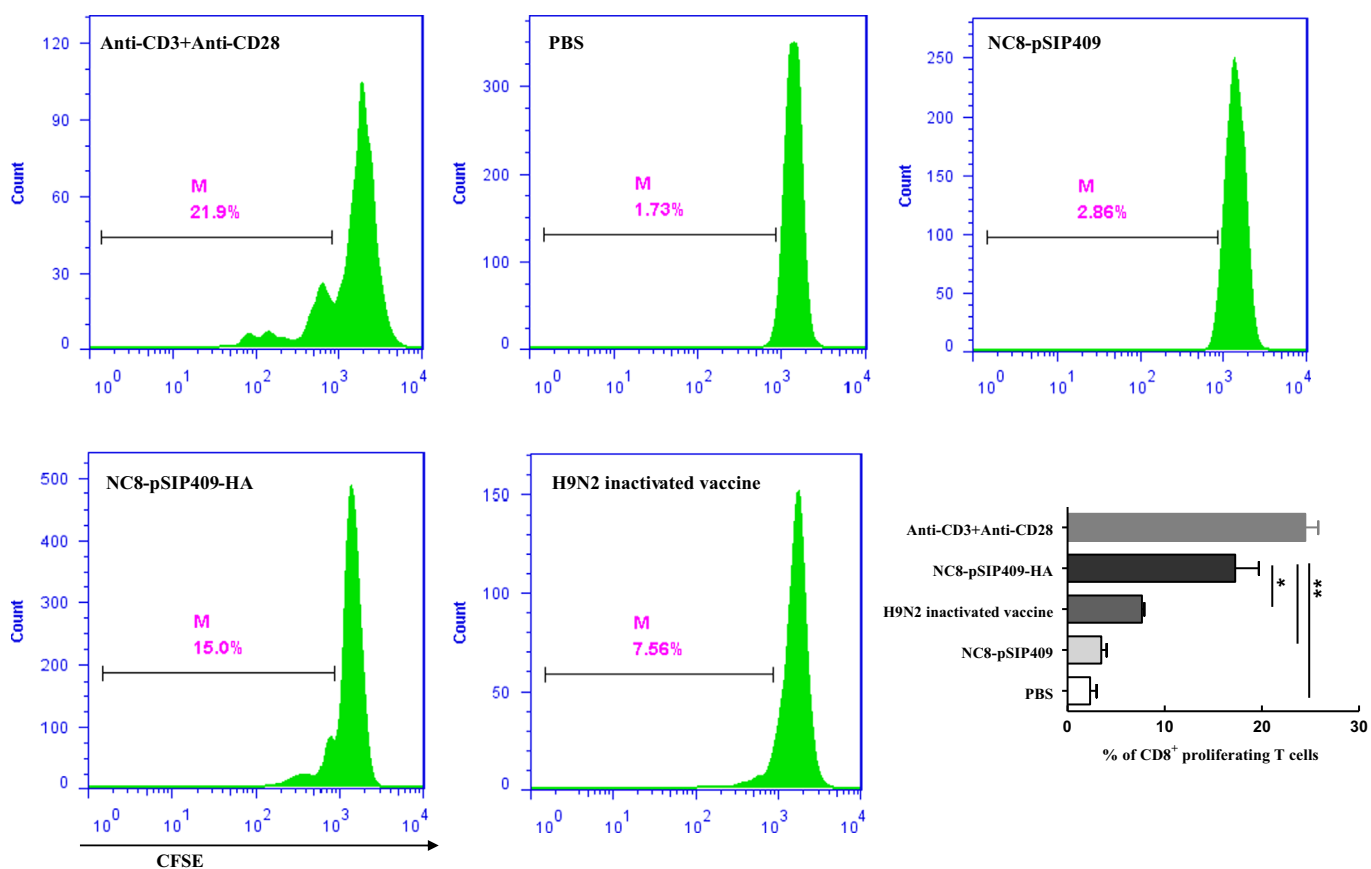
Fig. 5. Avian influenza virus (AIV) HA-specific T cell response to recombinant *Lactobacillus plantarum*. Splenocytes were isolated 30 days after the final immunization, stained with CFSE and stimulated in vitro with 10 µg/ml of purified HA protein for 3 days. The data shown are expressed in CFSE histograms of fluorescence intensity versus the number of fluorescing cells, indicating the percentage of the cell population positive for the CD8 antigen. The values in the figure are percentages of CD8$^+$ proliferating T cells. The immunization conditions are displayed at the top. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (**$P < 0.01$) and to the H9N2 inactivated vaccine (*$P < 0.05$). Data are expressed as the mean $\pm$ SEM of triplicate experiments. $n = 3$ mice per group.

inducing serum IgG antibodies (Lei et al., 2010) and IgA in the lungs and bronchoalveolar lavage fluids (BALF) (Kikuchi et al., 2014; Salva et al., 2010), sublingually administered *Lactobacillus rhamnosus* appears to be more effective for inducing protection against influenza virus infection, most likely as a result of the high elicited levels of anti-influenza virus-specific IgA in the lungs (Lee et al., 2013). In this study, the oral administration of recombinant *L. plantarum* NC8-pSIP409-HA can promote the formation of GCs within the mucosal and systemic sites in mice through increased numbers of FAS$^+$ PNA$^+$ B220$^+$ B cells in the PP, MLN and spleen. Higher levels of HA-specific IgA were also produced in fecal matter and BALF. Such secretory IgA antibody responses seem to play a role in preventing the entry and replication of the influenza virus in the respiratory tract; however, the mechanisms underlying the reductions in respiratory tract infections and other symptoms remain unclear.

Glycosylation of viral envelope proteins is very important and influences the immunogenicity and the sensitivity of the virus to neutralizing antibodies. Oligosaccharides may hinder proteolytic degradation, and with that affect, T-cell recognition by keeping the appropriate conformation of proteins (Bolmstedt et al., 2001; Li et al., 2008). Removal of glycans from influenza viral envelope proteins has been shown to enhance T cell response (Ertl and Ada, 1981; Wood and Elliott, 1998), and interfere with their immunogenicity (Sjölander et al., 1996). Presumably, sugars do not coat HA in *Lactobacillus*. In this study, the oral administration of recombinant NC8-pSIP409-HA elicited the strongest cellular immune response in the MLN and spleen. Glycosylation influences the delivery or processing of recombinant proteins for MHC class I

presentation and may block peptide binding to MHC class I (Haurum et al., 1995). In contrast, the serum IgG titer (Fig. 3C) is not particularly high compared to many vaccination regimens. Those phenomena implied that glycosylation of HA is very important for maintaining the level of humoral immunity. More importantly, our results support the previous findings that IFN-γ producing CD8$^+$ T lymphocytes induce protective immunity against natural influenza illness in humans (Sridhar et al., 2013). IFN-γ is a cytokine that is secreted by T lymphocytes and NK cells and leads to increased phagocytic activity, which effectively kills pathogens, modulates chemotaxis and up-regulates antigen presentation to induce Th1 responses against pathogen challenge (Schroder et al., 2004). Moreover, it is interesting that CD8$^+$ T cells have responded to HA 30 days after immunization. These strong T cell responses may improve the direct lysis of MHC class I-bearing infected cells (Lu et al., 2011). The effector/memory CD8$^+$ T cells at mucosal effector sites are notable because they participate in the initial AIV replication and subsequent viral dissemination. Most of the results indicated that T cellular immune responses are considered to play a key role in protecting immunity against AIV infection in mice.

As shown in Fig. 6, administration of bacteria alone appears to have improved the survival and weight loss from influenza infection. The use of probiotics to combat influenza has been shown previously to have some promise in protection against influenza. Indeed, oral administration of this bacterial strain (*Lactobacillus planarium*) has been shown to increase protection against influenza infection by itself (Kikuchi et al., 2014). In addition, specific *Lactobacillus* strains are also supposed to exert
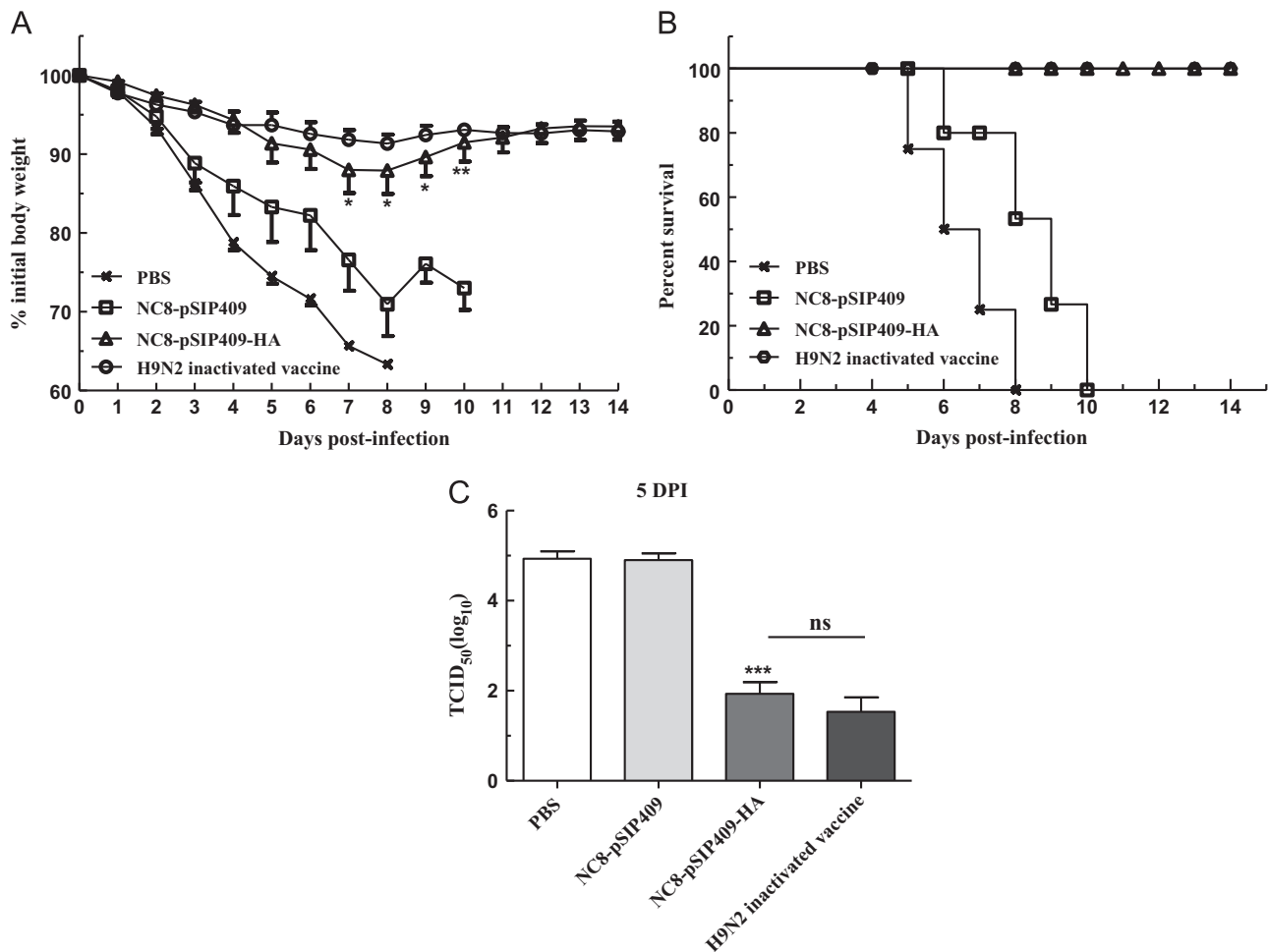
Fig. 6. Recombinant NC8-pSIP409-HA protected mice from the mouse-adapted H9N2 AIV infection. Six weeks after the final immunization, the animals were challenged intranasally with a lethal dose ($10 \times LD50$) and monitored for weight loss and percent survival. (A) Weight loss (%) of the mice after infection. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (*$P < 0.05$, **$P < 0.01$). Data are expressed as the mean $\pm$ SEM of triplicate experiments. $n=5$ mice per group. (B) Percent survival of mice 0–14 days after infection. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (*$P < 0.05$, **$P < 0.01$). Data are expressed as the mean $\pm$ SEM of triplicate experiments. $n=5$ mice per group. (C) For virus titration, lungs were harvested at 5 days after infection and homogenized. The amount of H9N2 virus in the supernatant was analyzed by TCID$_{50}$. *Statistically significant differences relative to the PBS and NC8-pSIP409 controls (***$P < 0.001$). Data are expressed as the mean $\pm$ SEM of triplicate experiments. $n=3$ mice per group.

beneficial health properties and are thus intensively studied for probiotic applications (Dicks and Botes, 2010; Doron and Gorbach, 2006). We therefore choose *L. plantarum* as a candidate vaccine vehicle. *L. plantarum* NC8, our model strain, has been confirmed to be particularly resistant to the conditions of the upper digestive tract in mice and chicken. Moreover, we observed that it is able to persist in the mouse intestine for more than 3–7 days, while *Lactococcus lactis* could not be detected in feces 24 h after ending of the feeding. Interestingly, in this study, the empty vector NC8-pSIP409 not induce B cell activation, we considered that the result may be associated with the immunizing doses and procedures, or the point in time of our detection.

Different vaccines and delivery modes were found to stimulate different immune responses. Inactivated pathogens, such as for influenza, are generally used for pathogens for which a humoral immune response is considered the primary protective immune response (Liu, 2010b). Subsequently, gene-based vaccines are able to deliver heterologous antigens into the antigen-processing pathways needed to stimulate MHC class I-restricted CTL responses (Liu, 2010a). In addition, probiotics-based vaccines are able to modulate immune stimulatory responses upon interaction with antigen-presenting cells such as dendritic cells (DCs) and macrophages. Several studies that show toll-like receptors (TLRs), play a key role upon the stimulation of DC and macrophages with recombinant *lactobacilli* (Kathania et al., 2013). Furthermore, exposure to *lactobacilli* induces the up-regulation of surface markers and production of several cytokines that modulate the function of DCs (Christensen et al., 2002; Weiss et al., 2011). It is well established that high IL-12 production by DCs matured by microbial stimuli induces Th1 polarization and thus strongly stimulates of the adaptive immune defense. In this study, the recombinant NC8-pSIP409-HA was more conducive to the protection of the integrity of the lungs, compared to the H9N2 inactivated vaccine, which may be because CD4$^+$ and CD8$^+$ Tregs increased significantly in the lungs of mice that received the oral administration of *L. rhamnosus* (Lee et al., 2013). Tregs represent suppressive cell subsets, which act to regulate T cell responses and are thereby, thought to prevent pathology resulting from excessive immune responses (Sakaguchi et al., 2008). These studies imply that probiotics enhance the activation of helper and cytotoxic T lymphocytes and beneficially modulate both innate and adaptive immunity.

In conclusion, the present results demonstrated that HA is an ideal target candidate for the development of vaccines against H9N2 AIV as previously reported, which can prevent the pandemic spread of the H9N2 influenza virus. Similar plans for H9N2 challenges in chickens are in progress pending regulatory approvals. Future studies are required to consider target AIV antigens to DC-targeting peptides; studies on AIV vaccination
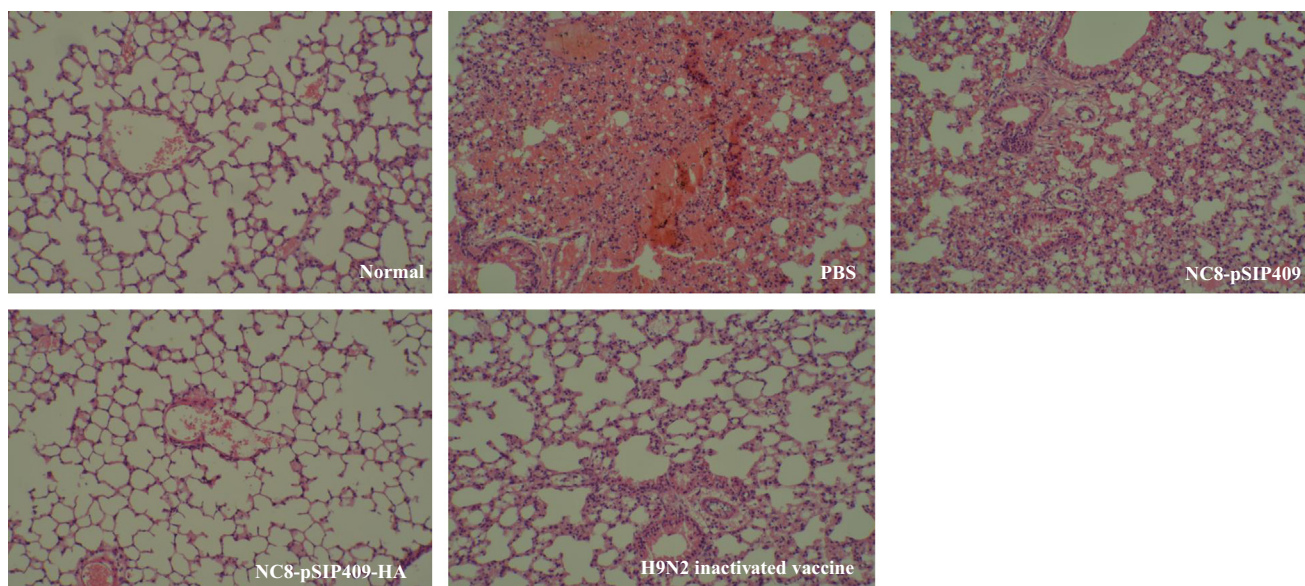
Fig. 7. Histopathological analysis of lungs in mice challenged with mouse-adapted H9N2 AIV. The mice were euthanized and the lung tissue samples were excised, fixed in 10% neutral buffered formalin and embedded in paraffin at 5 days post-challenge with the H9N2 viruses. Tissue sections were stained with hematoxylin and eosin (magnification, × 100).

should also address this possibility and determine the protective effect against heterologous virus challenges that model the natural exposure to viruses among birds.

Materials and methods

Construction of recombinant NC8-pSIP409-HA expressing HA gene

The *Escherichia coli-Lactobacillus* shuttle vector pSIP409 and *L. plantarum* NC8 (for the delivery of HA protein) were kindly provided by A.Kolandaswamy (Madurai Kamaraj University, India). The HA gene fragment (1704 bp) was re-amplified from previously constructed pGEM-T-HA following standard PCR conditions using the primer pairs with *Kpn*I and *Hind*III restriction sites underlined (forward primer: 5′ CGCGGTACCATGGAAACAATACACTAAT 3′, reverse primer: 5′ GGGAAGCTTTTATATACAAATGTTGCATCTGC 3′) at their 5′ end, respectively. The resulting amplicon was digested with *Kpn*I, and the 5′ termini were blunted using S1 nuclease treatment. The gene was then digested with *Hind*III to produce a sticky end at the 3′ end. Moreover, the plasmid vector pSIP409 was digested with *Nco*I and made blunt using S1 nuclease treatment, followed by digestion with *Hind*III to create the respective compatible ends. Purification of the linearized plasmid and the gene fragment were performed using a gel extraction kit (Axygen). The gene and the plasmid vector were ligated using a Quick Ligation Kit (Roche) and transformed into *E. coli* DH5α (Takara). The clones were confirmed by DNA sequencing using the primer, 5′CGCCCGGTTTAATTTGAAAATTGATATTAGCG3′. Finally, the pSIP409 plasmid and the recombinant pSIP409-HA plasmid were transformed into *L. plantarum* NC8 by electroporation as previously described (Kolandaswamy et al., 2009).

All the *L. plantarum* NC8 were cultured in De Man, Rogosa and Sharpe (MRS) medium at 30 °C without shaking. Erythromycin was used at a concentration of 10μg/ml. *E. coli* DH5α was cultured in Luria-Bertani (LB) medium containing 200 μg/ml of ampicillin.

Animals and ethics statement

Pathogen-free female BALB/c mice, aged from 6 to 8 weeks, were obtained from Beijing HFK Bioscience Co., Ltd., China and were reared with formulated commercial feed and water provided ad libitum throughout the whole experimental period. All experimental procedures and animal management procedures complied with the requirements of the Animal Care and Ethics Committees of Jilin Agriculture University. The animal facility of the Jilin Agriculture University is fully accredited by the National Association of Laboratory Animal Care.

Immunoblotting

To examine HA antigen expression levels, the recombinant NC8-pSIP409 and NC8-pSIP409-HA were cultured in MRS broth supplemented with 10 μg/ml erythromycin. Sakacin P (SppIP) was added to the culture medium at 0.3 of an OD_{600} to a final concentration of 50 ng/ml to induce antigen expression. After induction at 30 °C for 10 h, the bacteria were centrifuged at 5000g for 5 min and resuspended in 1 ml of TBS buffer (0.8% NaCl, 0.02% KCl, 25mM Tris, pH 7.4) containing 0.1 m Mphenyl-methylsulphonyl fluoride (PMSF). In brief, the harvested cells incubated at 30 °C for 5 min and disrupted on ice with sonication. The protein concentration of the culture lysates was then determined. Proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membranes. The presence of the HA protein was detected using monoclonal mouse anti-HA antibody followed by affinity-purified horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin G (Sigma). Visualization of the immunobinding was conducted by enhanced chemiluminescence (ECL) using an ECL Plus detection kit (Thermo Scientific).

Virus and homologous recombination HA protein

A H9N2 avian influenza virus A/duck/Xuzhou/07/2003(H9N2) was propagated in the allantoic cavity of 9-day-old chicken eggs. The allantoic fluid was harvested 72 h after inoculation, and virus in the allantoic fluid was titrated using a standard hemagglutination test as previously described (Hirst, 1942). The homologous rHA was from A/duck/Xuzhou/07/2003(H9N2) that has been expressed and purified in the company (Sino Biological Inc.) and used in all antibody and T cell assays.

Adaptation of wild-type H9N2 virus to increase virulence in mice

Wild-type H9N2 virus (A/duck/Xuzhou/07/2003) was adapted to growth in mice using a published protocol (Ilyushina et al., 2010). Briefly, female 8-week-old BALB/c mice were inoculated intranasally under sodium pentobarbital with virus. The lungs were harvested after 2 days and homogenized, and 50 µl of the centrifuged homogenate was used to inoculate the next passage. After a total of 9 passages, the virus present in the lung homogenate was passaged once in the allantoic cavities of 9-day-old chicken eggs at 37 °C for 72 h to prepare a virus stock.

Immunization and challenge

Eighty BALB/c mice were randomly divided into four groups. Four groups of mice were orally immunized with NC8-pSIP409-HA, NC8-pSIP409 and PBS by using oral gavage and H9N2 inactivated vaccine (Weike Biotechnology) as the positive control (intramuscular injection with 50 µl/mouse). Briefly, NC8-pSIP409-HA and NC8-pSIP409 were cultured at 30 °C in MRS broth supplemented with erythromycin (10 µg/ml) and SppIP (50 ng/ml) for 18 h in tightly capped flasks without shaking. Freshly cultured bacteria were harvested, washed twice with PBS and suspended in 200 µl of PBS containing 1×10^9 cfu per mouse. Administration via the oral gavage was repeated three times on a weekly basis throughout the study and the groups of mice were boosted twice two weeks later. Blood was drawn on days 21, 35 and 49 for antibody analyses. Six weeks after the final immunization, groups of BALB/c mice were challenged intranasally (i.n.) with $10 \times LD_{50}$ of mouse-adapted H9N2 virus under anesthesia induced intraperitoneally (i.p.) with 100 µl of 15 mg/ml Mebubarbital (Avertin; Sigma). Mice challenged with mouse-adapted H9N2 virus were monitored for 14 days with weight checks every day. For virus titration in the lung, viruses were inoculated into MDCK cells and cultured for 3–5 days, and $TCID_{50}$ values were measured as previously described (Bai et al., 2011). The experiments were carried out at least thrice.

HI assay

Hemagglutination inhibition (HI) antibody titers in the sera were determined using chicken erythrocytes and a standard HI microtiter assay as previously described (Haan et al., 2001). The HI titers were defined as the highest serum dilution capable of preventing hemagglutination.

Sampling from BALF and feces

BALF was obtained as previously described (Grangette et al., 2001). In brief, BALF was obtained postmortem by inserting a nylon cannula into the exposed trachea, which was tied in place. A hypodermic needle and syringe were attached and used to inject and withdraw 400 µl of 1 mM PMSF (Roche Diagnostics, Rotkreuz, Switzerland) in PBS thrice. The fluid samples were retained on ice before centrifugation at 4000g for 20 min at 4 °C, and the supernatants were then stored at -20 °C until analysis.

Fresh fecal samples were collected from groups of mice; 0.2 g fecal pellets were added to 1 ml PBS containing 1% BSA and 1 mM PMSF. After incubation at 4 °C for 12 h, the tubes were vortexed to disrupt all solid material and then centrifuged at 16,000g for 10 min. The supernatants were collected and stored at -20 °C until tested.

Ab assays

To detect the amount of each IgA and IgG Ab against HA, we carried out a protocol described previously based on the enzyme-linked immunosorbent assay (ELISA) (Haan et al., 2001). Briefly, 96-well polystyrene microtiter plates were coated with 1 µg of rHA (Sino Biological Inc.) using carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were washed thrice with washing buffer (15 mM PBS containing 0.1% Tween 20; PBST) and blocked with blocking buffer (3% bovine serum albumin (BSA) in PBST) at 37 °C for 1 h. The serum samples were added at two-fold dilutions from 1/2 to 1/1024 in wash buffer and incubated at 37 °C for 1 h. Following the incubation, the plates were washed four times with PBST, and anti-mouse-HRP conjugate (Southern Biotech) was added to all the wells at a dilution of 1:8000 and incubated for 1 h. After washing four times with wash buffer, the plate was developed with 0.02% *O*-phenylenediamine and 0.015% H_2O_2 (Zymed) in substrate buffer (15 mM citrate buffer pH 5.6), and the reaction was stopped after 10 min with 2N H_2SO_4. The absorbance was read at 492 nm. End point titers were defined as the highest dilution that gave an absorbance thrice higher than background for serum samples or twice for fecal and BALF samples.

Preparation of single-cell suspensions from PPs, MLNs and spleen

PP cells were separated from groups of mice using a published protocol (Kikuchi et al., 2014). Briefly, BALB/c mice were sacrificed by cervical dislocation after immunization. PP cells were collected from the side of the small intestine and incubated in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) with 10% FCS (Sigma-Aldrich) and 0.2 units of collagenase type I (1 mg/ml; Sigma-Aldrich) with a magnetic stirrer at 37 °C for 60 min. The cell suspensions were then passed through a 70-µm nylon cell strainer (BD Biosciences, San Jose, CA, USA), centrifuged at 4 °C for 5 min and washed with PBS. Then, they were vigorously shaken for 10 s and centrifuged at 4 °C for 5 min. After washes, the cells were incubated in FACS buffer for staining.

MLNs cells and splenocytes were isolated by gentle crushing of organs through a 70 µM pore filter (BD Falcon). Splenocytes were further treated with ACK lysis buffer (BD Pharm Lyse™) to lyse red blood cells (RBCs), and the cell suspensions were washed and resuspended in complete RPMI 1640.

Flow cytometry

Flow cytometry was carried out as described previously (Lu et al., 2011). In brief, single cell suspensions from immunized mice were blocked with CD16/CD32 (2.4G2, BD Pharmingen) and washed in fluorescence activated cell sorting (FACS) buffer (PBS, 1% FCS, 0.09% sodium azide). The cells were analyzed for B cell activation by staining with phycoerythrin (PE)-conjugated antibodies for B220 (clone RA3-6B2, BD Pharmingen), PE-Cy7-conjugated antibodies for Fas (clone Jo2, BD Pharmingen) and fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA, Sigma). After staining, the samples were examined with a BD FACS Calibur (BD Biosciences), and the data were analyzed by using FlowJo 7.6.1 software.

Intracellular cytokine staining

To estimate cytokine-producing cells, intracellular cytokine staining (ICS) was performed as previously described (Lee et al., 2007). Briefly, MLN and spleen lymphocytes from the different groups of mice were isolated after booster vaccination and incubated for 8 h with HA antigen (10 µg/ml) or medium alone.

The cells were then cultured for another 4 h in the presence of monensin (10 μg/ml; BD GolgiStop™ protein transport inhibitor). The cells were washed and stained with PerCP-Cy5.5-conjugated antibodies for CD3e (clone 145-2C11), APC-Cy7-conjugated antibodies for CD8 (clone 53-6.7) (BD Pharmingen). The cells were then washed, fixed and permeabilized with the Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit (BD Pharmingen) according to the manufacturer's instructions. Finally, cells were stained with either APC-conjugated anti-IFN-γ (clone XMG1.2) or isotype control APC conjugated antibodies (BD Pharmingen). The cells were examined using BD FACS Canto™ flow cytometry.

T cell proliferation assay

T cell proliferation in response to HA antigen was evaluated as described previously (Lu et al., 2011). In brief, carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) was added to splenocyte suspensions by immunizing mice with 10^7/ml in pretreated PBS (37 °C) containing 0.1% BSA to a final concentration of 2 μM. The reaction mixtures were then incubated for 15 min at 37 °C. Then, cold FBS was added and incubated on ice for 10 min to stop the reaction, and the samples were washed thrice with RPMI-1640 containing 10% FCS. After labeling with CFSE, the splenocytes (5×10^5) were plated into 96-well plates and cultured in 200 μl of complete RPMI medium with or without HA (10 μg/ml; Sino Biological Inc.), anti-CD3 (0.1 μg/ml) plus anti-CD28 (2 μg/ml) as a positive control. After 3 days, the cells were collected and stained with PE-Cy7-conjugated antibodies for CD4 (clone GK1.5) and APC-Cy7-conjugated antibodies for CD8 (clone 53-6.7), and then analyzed by BD FACS Canto™ flow cytometry.

IFN- γ ELISpot assay

The IFN-γ ELISpot assay was performed 21 days after the final immunization using an ELISpot kit for mouse IFN-γ as recommended by the manufacturer (Mabtech). Briefly, first the membrane of the ELISpot plate was pre-wetted by adding 50 μl 70% ethanol per well in sterile conditions. The mouse IFN-γ plate was filled with 200 μl/well of sterile culture media and incubated for at least 30 min at room temperature. After aspirating the culture media from the wells, 100 μl of 5×10^5 splenocytes and MLN cells were added per well. HA protein (10 μg/ml) was used as stimuli in a 37 °C humidified incubator with of 5% CO_2 for 48 h. Control wells were not stimulated with HA protein. After incubation, each well was aspirated and washed, the plates were treated sequentially with biotinylated monoclonal anti-mouse IFN-γ antibody, streptavidin-horseradish peroxidase and the substrate solution to reveal the spots. The developed microplate could be inspected by counting spots in an ELISpot reader (AID).

Histopathological examination

To assess pulmonary inflammation after mouse-adapted H9N2 virus infection, mouse lungs were isolated from each group and immediately placed in a 10% neutral buffered formalin fixative. The tissues were then processed and embedded in paraffin. Slides were then cut, the sections were stained with hematoxylin and eosin to visualize cellular inflammation, and the samples were read blindly.

Statistical analysis

All results are expressed as geometric mean titers with mean of at least three independent experiments. Statistical significance was determined using unpaired two-tailed t tests. GraphPad Prism 5 software was used for the statistical analyses.

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