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Research Paper

Molecular mechanisms underlying protection against H9N2 influenza virus challenge in mice by recombinant *Lactobacillus plantarum* with surface displayed HA2-LTB

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ABSTRACT

It has been considered that the Avian influenza virus (AIV) causes severe threats to poultry industry. In this study, we constructed a series of recombinant *Lactobacillus plantarum* (*L. plantarum*) with surface displayed hemagglutinin subunit 2 (HA2) alone or together with heat-labile toxin B subunit (LTB) from enterotoxigenic *Escherichia coli*. Balb/c mice were used as model to evaluate the protective effects of recombinant *L. plantarum* strains against H9N2 subtype challenge.

The results showed that the presence of LTB significantly increased the percentages of $CD3^+CD4^+IL\text{-}4^+$, $CD3^+CD4^+IFN\text{-}\gamma^+$ and $CD3^+CD4^+IL\text{-}17^+$ T cells, as well as $CD3^+CD8^+IFN\text{-}\gamma^+$ T cells in spleen and MLNs determined by Fluorescence-Activated Cell Sorting assay. Similar increased production of serum IFN-γ was also confirmed by enzyme linked immunosorbent assay (ELISA). The *L. plantarum* with surface displayed HA2-LTB also dramatically increased the percentages of $B220^+$ IgA^+ B cells in peyer patch, in consistent with elevated production of mucosal SIgA antibody determined by ELISA. Finally, the orally administrated HA2-LTB expressing strain efficiently protected mice against H9N2 subtype AIV challenge shown by increased survival percentages, body weight gains and decreased lung lesions in histopathologic analysis. In conclusion, this study provides more detail mechanisms underlying the adjuvant effects of LTB on heterologous antigen produced in recombinant lactic acid bacteria.

1. Introduction

Avian influenza virus (AIV), belonging to the family of *Orthomyxoviridae* with enveloped membrane and segmented negative-stranded RNA, has caused severe economic loss in field production and resulted in public concerns about the transfer from poultry to human beings. AIV is classified to 16 hemagglutinin (HA) subtypes (H1-16) and 9 neuraminidase (NA) subtypes (N1-9) based on the displayed antigenic variation on virus surface, resulting in a total of 144 subtype combinations. In particular, the low pathogenic virus H9N2 subtype strains have been demonstrated to be the main circulated virus in domestic poultry production of China recently, especially in the north of China (Bi et al., 2016).

To secure poultry production, vaccination using the inactivated vaccines or live recombinant vaccine has been exercised extensively during the last several decades. However, more efficient vaccines are still in urgent need since that a number of disadvantages are also noticed in the egg-based inactivated AIV vaccines, such as the long production cycle, the limited production capacity, and the inability to provide complete protection against virulent field challenge, allowing a small number of infected chicken to excrete virus to environment without obvious clinical signs, which results in the long-term circulation of AIV in field. To enlarge the list of possible options for AIV vaccination, a number of new concept vaccines such as subunit vaccine

Abbreviations: AIV, avian influenza virus; L.plantarum, lactobacillus plantarum; HA2, hemagglutinin subunit 2; LTB, heat-labile toxin B subunit; ELISA, enzyme linked immunosorbent assay; HA, hemagglutinin; NA, neuraminidase; VLPs, virus like particles; KLH, keyhole limpet hemocyanin; ETEC, enterotoxigenic *Escherichia coli*; GRAS, generally regarded as safe; LAB, lactic acid bacteria; pgsA, poly-γ-glutamic acid synthetase A; PGA, polyglutamate synthetase system; dmLT, double mutant heat labile toxin; CT, cholera toxin; PBMCs, peripheral blood mononuclear cells; E.coli, *Escherichia. coli*; LB, luria-bertani medium; erm, erythromycin; PBS, phosphate buffered saline; MLNs, mesenteric lymph nodes; BWG, body weight gain; HE, hematoxylin-eosin; TMB, tetramethylbenzidine

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(He et al., 2014), DNA vaccine (Kong et al., 2012), virus like particles (VLPs) (Zheng et al., 2016) and reverse genetics vaccines have been described extensively recently.

The glycoprotein HA has been investigated detailly in vaccine strategy due to its significant roles in host-virus interaction. The HA protein consists of a globular HA1 domain and a more conserved HA2 stem. Although the high variation in HA protein has been noticed, there are still some conserved sequences in HA protein, especially in HA2 subunit. The N-terminal fusion peptide of HA2, especially the first 11 residues, has been involved in vaccine design against AIV infection which are effective in inducing neutralized antibodies across different HA subtypes. However, since the HA2 protein is partially hidden inside the virus and thus poorly immunogenic, it will be necessary to increase its immunogenicity through additional approaches, such as the conjunction with keyhole limpet hemocyanin (KLH) (Gong et al., 2016) and trimeric expression (Impagliazzo et al., 2015). Heat-labile toxin B subunit (LTB) from enterotoxigenic *Escherichia coli* (ETEC) has been proved to be an effective vaccine adjuvant in a number of studies, stimulating enhanced humoral and cellular immune responses which provide significant protection against challenge with both virus and pathogens (Liu et al., 2016; Sharma and Dixit, 2016; Zhang et al., 2016).

As one of the most popular GRAS (Generally Regarded As Safe) organisms (Garcia-Fruitos, 2012), lactic acid bacteria (LAB) represents a promising vector to deliver protective antigens (Chowdhury et al., 2014; Morello et al., 2008; Nouaille et al., 2003; Tauer et al., 2014), yielding orally administrated vaccines which will benefit domestic animal production compared to traditional injection relied immunization approach. LAB belong to a clade of Gram-positive, non-sporeforming, acid-tolerant bacteria which has been demonstrated to benefit host, such as the improvement of host immunity and control of infectious disease (Hosoya et al., 2011; Perdigon et al., 2001; Perdigon et al., 2002). The productions of heterologous proteins are driven by either constitutive promoter (Borrero et al., 2011; Tauer et al., 2014) or inducible promoter, such as nisin (Kong et al., 2017; Kong et al., 2016; Kong and Lu, 2014) and SppIP system (Jiang et al., 2015; Yang et al., 2015). The synthesized proteins were classified into cytoplasmic, cell wall anchoring and secretion expression according to the different positions where proteins are synthesized (Michon et al., 2016). Compared with cytoplasmic and secreted expression, the cell wall anchoring approach is generally considered to be more efficient to stimulate robust host immune response since the cell wall associated protein could resist harsh conditions and increase its on-site concentration (Michon et al., 2016). Poly-γ-Glutamic Acid Synthetase A (pgsA), which is a constituent protein of polyglutamate synthetase system (PGA) of Bacillus subtilis, has been widely used to anchor foreigner protective antigens on the surface of lactic acid bacteria (Lei et al., 2015; Yoon et al., 2012). In a previous study (Cai et al., 2016), we have established a shorten version of pgsA, named pgsA', which was found to dramatically increase the expression level of selected proteins in vitro, indicating its promising application ability in vaccine study.

In this study, we took use of *Lactobacillus plantarum* (*L. plantarum*) to display the conserved HA2 region of H9N2 subtype AIV either alone or together with LTB on cell surface by pgsA' anchoring sequence and evaluated its induced immunological responses after oral administration in Balb/c mice model. The results showed that the HA2-LTB fusion construction significantly increased the production of antibodies and IFN-γ, providing at least partial protection against AIV challenge.

2. Methods

2.1. Bacteria strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia. coli* (*E. coli*) Top10 cells were grown in Luria-Bertani medium (LB) at 37 °C with shaking and *L. plantarum* NC8 cells (Thea Aukrust, 1992) were grown aerobically in MRS medium at 30 °C without shaking. When necessary, solid media were prepared by adding 1.5% (w/v) agar to the broth. When required, ampicillin was added 200 μg/mL for *E.* coli, erythromycin (erm) was added as follows: 200 μg/mL for *E. coli* and 10 μg/mL for *L. plantarum.*

2.2. DNA manipulation

The coden optimized HA2 gene of H9N2 subtype AIV and HA2-LTB fusion operon for better expression in *L. plantarum* were synthesized by Genewiz company (Suzhou, China) and cloned into pUC57 vector. The fragments were then digested with XbaI/HindIII and ligated with pSIP409-pgsA' digested with the same enzymes, yielding pSIP409-pgsA'-HA2 and pSIP409-pgsA'-HA2-LTB, respectively.

2.3. Western blotting assay

Each strain was cultured overnight and inoculated into MRS medium with 10 μg/mL Erm at the ratio of 1:100 and incubated further at 30 °C until OD600 = 0.3. After that, the inducer pheromone SppIP was added to the final concentration of 50 ng/mL for additional 5 h. The cells were then collected by centrifugation at the speed of 12,000 rpm for 5 min and resuspended with PBS buffer, which were subjected to sonic disruption. The cell-free extract was then collected by centrifugation and separated with 12% sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE), followed by western blotting assay. The mouse anti-HA monoclonal antibody (Sino Biological Inc. Beijing, China) and mouse anti-LTB monoclonal antibody (Abcam Ltd, Shanghai, China) were used as primary antibodies, respectively, followed by HRP-labeled goat anti-mouse IgG antibody (Cwbiotech, Beijing, China). The ECL substrates (Thermo Scientific Pierce) were then used to observe the immunoreactive bands under Amersham WB (GE healthcare, USA).

2.4. Animal experiments

6-7 weeks old pathogen-free Balb/c mice were purchased from Beijing HFK Bioscience Co., Ltd., China. A total of 44 mice were random divided into 4 groups with 11 mice each group, subjected to either phosphate buffered saline (PBS), NC8(pSIP409-pgsA'), NC8(pSIP409-pgsA'-HA2) or NC8(pSIP409-pgsA'-HA2-LTB), respectively. In detail, strains were cultured

cultured at 30 °C in MRS with erythromycin (10 μg/mL) and then induced with 50 ng/mL of SppIP-inducing peptide overnight. After washing twice with PBS, the fresh bacteria were collected and resuspended with PBS. The mice were orally immunized with recombinant NC8 strains with individual plasmid at the dose of 1.0×10^9 CFU/0.1 mL PBS for continuous 3 doses in the first week. After one week interval, the mice were boosted twice at week 3 and 5, respectively. Then 3 mice from each group were necropsied at week 6, the serum and intestines washing samples were collected for antibody determination, whereas the single cell suspensions from spleen, mesenteric lymph nodes (MLNs) were collected and subjected to fluorescence activated cell sorting (FACS) as described below. The rest mice were then challenged with mouse adapted H9N2 subtype AIV at the dose of 0.05 ml $10 \times LD_{50}$ of H9N2 by nasal drops and the clinical signs, body weight gain (BWG) and survival rates were observed and recorded during the next 14 days. The lung samples from survival mice were then collected for hematoxylin-eosin (HE) staining and histopathologic analysis.

2.5. Determination of HA specific serum IgG and intestinal SIgA antibody

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the HA specific serum IgG and intestinal SIgA antibodies as described before (Li et al., 2008) with minor modifications. In detail,