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Sublingual administration of *Lactobacillus rhamnosus* affects respiratory immune responses and facilitates protection against influenza virus infection in mice

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SUMMARY

The extensive morbidity and mortality caused by influenza A viruses worldwide prompts the need for a deeper understanding of the host immune response and novel therapeutic and/or prophylactic interventions. In this study, we assessed the sublingual route as an effective means of delivering probiotics against influenza virus in mice. In addition, IgA levels, NK cell activity, T cell activation, and cytokine profiles in the lungs were examined to understand the mechanism underlying this protective effect. Sublingual administration of *Lactobacillus rhamnosus* provided enhanced protection against influenza virus infection by enhancing mucosal secretory IgA production, and T and NK cell activity. Moreover, interleukin (IL)-12 levels in the lungs increased significantly. Conversely, IL-6 and tumor necrosis factor alpha levels in the lungs decreased significantly. On the basis of these promising findings, we propose that the sublingual mucosal route is an attractive alternative to mucosal routes for administering probiotics against influenza virus.

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1. Introduction

Influenza virus infection is a significant cause of morbidity and mortality all over the world. In particular, elderly individuals and young children are at increased risk due to poor or weakened immune function. Vaccination can help protect against the prevalent subtypes of influenza, but new subtypes represent a global pandemic threat. Furthermore, emerging resistance to neuraminidase inhibitors is a serious concern. Therefore, enhancing natural defense mechanisms by triggering nonspecific cell-mediated immunity in a host might be a practical effective method for managing influenza virus infections.

Lactobacilli are reported to have beneficial effects on host homeostasis and to be effective in activating the immune system (Fernandes and Shahani, 1990; Gilliland, 1989). In previous studies, Lactobacilli were found to be effective in the prevention or treatment of influenza virus infection in mice (Kobayashi et al., 2011; Maeda et al., 2009; Yasui et al., 2004). In particular, intranasal administration of lactobacilli is suggested to effectively protect against respiratory infection by directly augmenting the respiratory immune system (Harata et al., 2010; Hori et al., 2001; Izumo et al., 2010; Kawashima et al., 2011; Youn et al., 2012). Although intranasal administration of lactobacilli is effective for preventing influenza virus infection in mice, safety concerns associated with the retrograde transport of immunogens to the central nervous system (CNS) have been raised (Armstrong et al., 2005; Lemiale et al., 2003).

It was recently demonstrated that the sublingual mucosa is an efficient site for the induction of broad-spectrum immune responses (Cuburu et al., 2007). Sublingual administration of live or inactivated influenza virus induced Ab and T cell responses in both the local mucosa of the respiratory tract as well as the systemic compartment, which yielded protection of mice from lethal infection (Song et al., 2008). Importantly, unlike the intranasal route, sublingual immunization does not redirect immunogens to the CNS (Neutra and Kozlowski, 2006; Song et al., 2008).

In this study, we examined the protective efficacy of the sublingual administration of live *Lactobacillus rhamnosus* against the influenza virus in mice. Moreover, to understand the mechanism underlying this clinical protective effect, we performed

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immunological assays including examination of immunoglobulin A (IgA) levels, natural killer (NK) cell activity, T cell activation, and cytokine profiles in the lung.

2. Materials and methods

2.1. Animals

Female specific pathogen-free (SPF) BALB/c mice (Orient Bio Laboratories, Seoul, Korea) weighing 18–20 g were used. All experiments were carried out in compliance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

2.2. Preparation and administration of L. rhamnosus

L. rhamnosus were cultured for 24 h at 37 °C in MRS broth (Difco Laboratories, Detroit, MI), harvested by centrifugation at 1,400*g* at 4 °C for 10 min, washed three times with saline and lyophilized. The lyophilized powder was suspended in distilled water for sublingual administration to mice.

2.3. Virus

Influenza A/NWS/33 (H1N1) virus was grown in the allantoic sacs of 11-day-old chicken embryos at 37 °C for 2 days. The allantoic fluid was harvested and stored at −70 °C until used. The titer of virus in the allantoic fluid was determined as the 50% egg infective dose (EID_{50}). Briefly, serial 10-fold dilutions of the allantoic fluid were injected into embryonated eggs, and the presence of the virus in the allantoic fluid of each egg was determined based on the hemagglutinating capacity of the virus. In challenge studies, mice were challenged intranasally with 100 μl of $10^{4.0}$ EID_{50} influenza virus after anesthesia with an intraperitoneal injection of Avertin (375 mg/kg).

2.4. Experimental design

2.4.1. Determination of optimum duration of sublingual administration of L. rhamnosus

Mice received sublingual administration of *L. rhamnosus* at a concentration of 1×10^8 colony forming units (cfu) per mouse for 3, 6, 10, 13 or 16 days. After administration, four mice in each group were sacrificed for IgA and IL-12 determination.

2.4.2. Determination of the dose-dependent effect of sublingual administration of L. rhamnosus

Animals were assigned to three experimental groups: the *L. rhamnosus*, placebo, and normal control groups. Mice received sublingual administration of *L. rhamnosus* at concentrations of 1×10^8, 1×10^7, or 1×10^6 cfu per mouse for 10 days before the viral challenge. Saline was also administered in the same manner and for the same time as the mice in the placebo group. This was performed to confirm that responses to saline were similar to the responses to placebo. The mice in the normal control group were treated with neither lactobacilli nor influenza virus. After challenge with influenza virus, survival rate and weight loss were observed daily for 14 days post-infection (p.i.) (30% loss in body weight as the IACUC endpoint).

2.4.3. Effects of sublingual administration of L. rhamnosus on the lung virus titer and immune response in the lungs

Mice were treated with 10^8 cfu *L. rhamnosus* per mouse for 10 days before challenge as described above. Four mice in each group were sacrificed on days 3 and 6 p.i. for IgA determinations and flow cytometric analysis, on days 2, 4, and 6 p.i. for cytokine analyses, and on day 4 p.i. for analysis of NK cell activity. Furthermore, four mice in each group were also sacrificed on days 3 and 6 p.i. and their lungs were removed, weighed, and assigned a consolidation score ranging from 0 (normal) to 4 (maximal consolidation), depending on the percentage of the lung exhibiting a typical plum coloration. Each lung was assayed for infectious virus titer.

2.5. Determination of IgA concentration

For the titration of anti-influenza virus IgA, a microwell plate was coated with 10^6 $TCID_{50}$ of A/NWS/33 (H1N1) virus in PBS buffer (pH 7.4). The plate was washed using washing buffer (50 mM Tris–HCl containing 0.05% Tween-20) and then blocked with PBS containing 1% BSA for 1 h at room temperature. The lung homogenates (100 μL per well) were added to each well and incubated for 1 h at room temperature. The plate was washed five times using washing buffer and then incubated with HRP-conjugated anti-mouse IgA monoclonal antibody (Bethyl, Montgomery, TX) for 1 h. The plate was washed five times with washing buffer and incubated at room temperature for 30 min in tetramethylbenzidine (TMB) solution (BD Biosciences) before the stop solution (2 N H_2SO_4) was added. The absorbance was measured at 450 nm on a microplate reader (Sunrise, Tecan, Switzerland).

2.6. Cytokine assay

Cytokine levels in the clarified lung tissue homogenates were determined using ELISA kits for mouse interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-6, IL-12, tumor necrosis factor-alpha (TNF-α), and MCP-1 according to the manufacturers' instructions in duplicate against a standard curve (R&D Systems, Minneapolis, MN).

2.7. Determination of lung virus titers

Each mouse lung was homogenized and centrifuged at 1400*g* at 4 °C for 20 min. The supernatants were 10-fold serially diluted with PBS. The infectivity of the virus in the supernatant was determined from the median tissue culture infective dose ($TCID_{50}$) by using Madin–Darby canine kidney (MDCK) cells.

2.8. NK cell activity in splenic cells

The effect of sublingual administration of *L. rhamnosus* on splenic NK cell activity in influenza virus-infected mice was examined by using a cytotoxicity assay kit (CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega KK, Tokyo, Japan) based on the modified method of (Urbanowicz et al., 2009). YAC-1 cells were used as the target cells for NK cell activity. Splenic cells as the effector cells were separated by density gradient centrifugation on a Histopaque-1083® (Sigma–Aldrich, St. Louis, MO). The effector (1×10^5 cells/well) and target cells (1×10^4 cells/well) were mixed at a ratio of 10:1, plated in quadruplicate on 96-well U-bottomed plates, and incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, the cultured mixtures in the 96-well plates were centrifuged, and the supernatants were incubated with the substrate solution provided with the kit in 96-well plates for 30 min at room temperature to detect lactate dehydrogenase (LDH) activity. Then the absorbance of each well was measured at 490 nm by a microplate reader. The percentage of specific release was calculated according to the manufacturers' instructions.

2.9. Flow cytometric analysis

The lungs, mediastinal lymph nodes, and spleens were minced and filtered through a cell strainer to create a single cell suspension preparation. Lymphocytes were isolated using Histopaque-1083® and stained with appropriate combinations of fluorescently labeled monoclonal antibodies, including fluorescein isothiocyanate (FITC)-labelled anti-CD4 (AbD Serotec, Oxford, UK) or anti-CD8 (AbD Serotec) and allophycocyanin (APC)-labeled anti-CD25 (eBioscience, San Diego, CA). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) with Cell QUEST software (BD Biosciences).

2.10. Statistical analyses

All results are expressed as the mean ± standard error of the mean (SEM). Significant differences among treatments were evaluated by 2-way ANOVA or Student's *t*-test where appropriate. Differences in the survival rate between the groups after lethal challenge with influenza virus were analyzed using the logrank test by the Kaplan–Meier method. *P*-values of less than or equal to 0.05 were considered statistically significant.

3. Results

3.1. Optimum duration of sublingual administration of L. rhamnosus

To determine the optimal duration of prophylactic administration, we compared the effect of sublingual administration of *L. rhamnosus* on IgA and IL-12 secretion according to the duration of administration. Interestingly, IgA (Fig. 1A) and IL-12 (Fig. 1B) levels peaked on the 10th day of administration and decreased gradually thereafter.

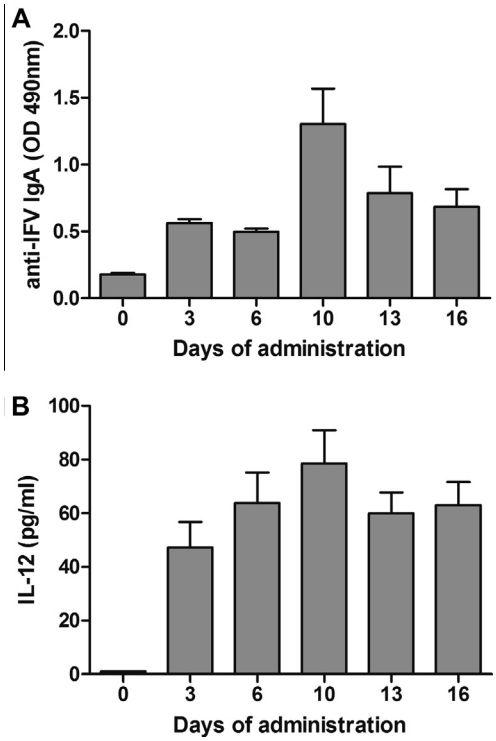

Fig. 1. Optimum duration of sublingual administration of *Lactobacillus rhamnosus* BALB/c mice were treated sublingually with *L. rhamnosus* (10^8 cfu per mouse) for 3, 6, 10, 13, or 16 days. Lung samples were collected and used to quantify IgA (A) and IL-12 (B) by ELISA. The assay detection limit was 19.5 pg/mL for IL-12.

3.2. Protection against influenza virus infection by sublingual administration of L. rhamnosus

We compared the protective efficacy of sublingual administration of *L. rhamnosus* according to the administration dose. In the mouse challenge study, administration of *L. rhamnosus* reduced mortality in a dose-dependent manner. The mice in the placebo group began to die at 7 days p.i. and the survival rate was 0% at 11 days p.i. (Fig. 2A). The survival rates of mice sublingually administered with *L. rhamnosus* at 10^8, 10^7, and 10^6 cfu/mouse were 70%, 50%, and 10%, respectively. The survival rates of the mice treated with 10^8 and 10^7 cfu/mouse were significantly different from that of the placebo group ($p < 0.001$ and $p < 0.05$, respectively). There was no significant difference in the mean change of body weight between the placebo group and *L. rhamnosus*-administered groups (Fig. 2B). At 3 and 6 days p.i., the lung lesion scores of the *L. rhamnosus*-administered mice were significantly lower than those of the placebo group ($p < 0.05$) (Table 1).

3.3. Effect of sublingual administration of L. rhamnosus on IgA levels, cytokine profile, and NK cell activity

Anti-influenza virus IgA concentrations in the lung homogenates from mice treated with 10^8 cfu of *L. rhamnosus* were significantly higher than that in the placebo group on day 6 p.i. (Fig. 3). Furthermore, IL-12 levels in the lung homogenates from the *L. rhamnosus*-administered mice were significantly higher than that in the placebo group on day 2 p.i. (Fig. 4A). TNF-α levels in the lung homogenates from mice treated with *L. rhamnosus* were significantly lower than those in homogenates from the placebo group on days 2 and 6 p.i. (Fig. 4B). The levels of IL-6 in the lung homogenates from mice treated with *L. rhamnosus* were also significantly lower than those in homogenates from the placebo group on days 2 and 4 p.i. (Fig. 4C). IFN-γ, IL-1β, and MCP-1 levels were not

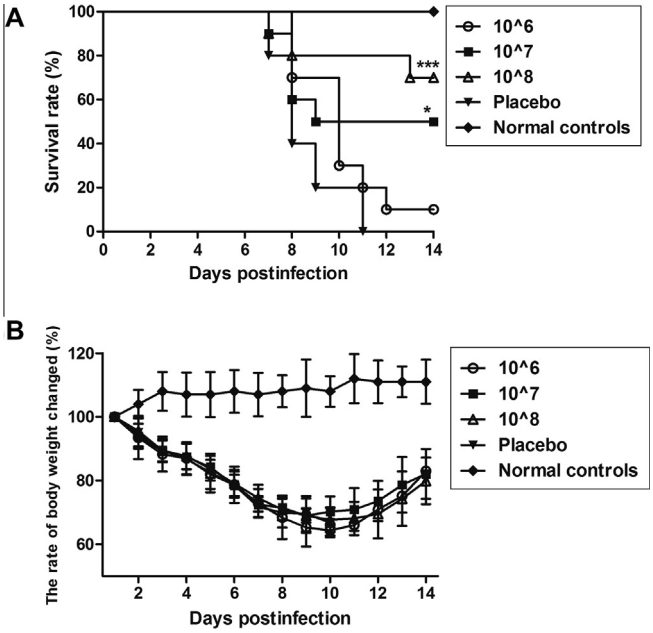

Fig. 2. Effect of sublingual administration of *L. rhamnosus* on survival rate and weight loss in mice challenged with influenza virus BALB/c mice were treated sublingually with *L. rhamnosus* (10^6–10^8 cfu per mouse) for 10 days before inoculation with the A/NWS/33 virus. The survival rate (A) and change in body weight (B) were daily monitored for 14 days. Treatments groups, $n = 10$; placebo group, $n = 10$; normal control, $n = 10$. Statistical analysis was performed by the log rank test according to the Kaplan–Meier method. Asterisks indicate significant differences ($^*p < 0.05$, $^{***}p < 0.001$) compared with the results in the placebo group.

Table 1
Effect of sublingual administration of *L. rhamnosus* on influenza virus replication in the lungs.

Group	Mean lung parameters					
	Day 3			Day 6		
	Lesion score[c] ± SEM	Lung weight (mg ± SEM)	Virus titer (log_{10}/g ± SEM)	Lesion score[c] ± SEM	Lung weight (mg ± SEM)	Virus titer (log_{10}/g ± SEM)
Placebo[a]	2.3 ± 0.1	185.0 ± 5.0	8.6 ± 0.6	4.0 ± 0.0	315.0 ± 23.3	7.4 ± 0.1
L. rhamnosus[b]	1.6 ± 0.1*	190.0 ± 5.8	8.2 ± 0.5	3.1 ± 0.1*	292.5 ± 6.3	6.7 ± 0.2

[a] Placebo group was not treated before virus challenge ($10^{4.0}$ EID_{50}/0.1 mL).
[b] Mice were treated with 10^8 cfu per mouse of *L. rhamnosus* for 10 days before challenge ($10^{4.0}$ EID_{50}/0.1 mL).
[c] The lungs were assigned a consolidation score ranging from 1 (normal) to 4 (maximal plum coloration).
* $p < 0.05$ compared to placebo group. Statistical significance was determined by Student's *t*-test.

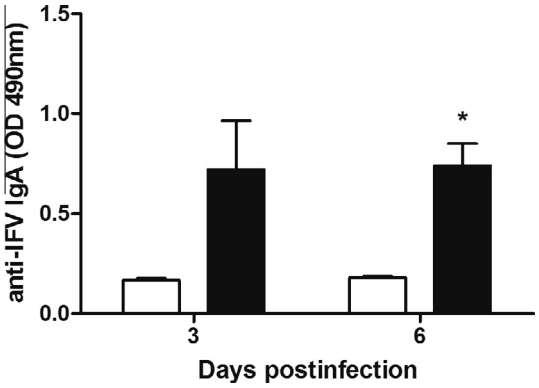

Fig. 3. Effect of sublingual administration of *L. rhamnosus* on anti-influenza virus IgA titers in the lungs of mice BALB/c mice were administered sublingually with *L. rhamnosus* (10^8 cfu/mouse) for 10 days before inoculation with the A/NWS/33 virus. The anti-influenza virus IgA titers in the lungs were determined by ELISA on days 3 and 6 p.i. Statistical significance was determined by Student's *t*-test. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$) compared with the results in the placebo group.

significantly different between the placebo and *L. rhamnosus*-administered groups (data not shown). At 4 days p.i., the NK cell activities of splenocytes in the *L. rhamnosus*-administered group were significantly higher than that in the placebo group ($p < 0.05$, Fig. 5). At 6 days p.i., CD25 expression by CD^{4+} lymphocytes was significantly higher in the lungs of mice in the *L. rhamnosus*-administered group than that in the normal control group (Fig. 6A). Interestingly, CD25 expression by CD^{8+} lymphocytes in the lungs of the *L. rhamnosus*-administered mice was significantly higher than that in the placebo group and the normal control group on day 6 p.i. (Fig. 6B). CD25 expression by CD^{8+} lymphocytes was also significantly higher in the mediastinal lymph nodes of mice in the *L. rhamnosus*-administered group than the normal control group (Fig. 6D).

4. Discussion

Mucosal immune responses are important in the first line of host defense because most microbial pathogens invade via mucosal surfaces (Neutra and Kozlowski, 2006). Oral mucosa including buccal, sublingual, and gingival mucosa have received attention as novel delivery sites for therapeutic drugs because they do not subject proteins and/or peptides to the degradation usually caused by gastrointestinal administration. Among the oral mucosal routes, the sublingual route is commonly used for immunotherapeutic treatments of allergy because antigens are absorbed quickly and allowed them to enter the bloodstream without passing through the intestine or liver, thereby eliciting allergen-specific tolerance (Kildsgaard et al., 2007). No cases of anaphylactic shock in humans were observed in recent studies of sublingually administered immunotherapy targeting allergies (Agostinis et al., 2005). These

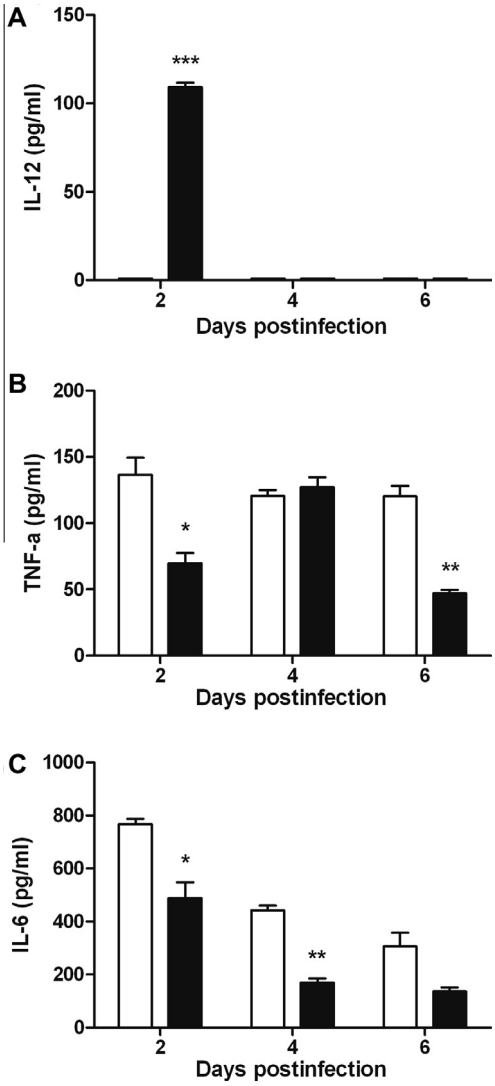

Fig. 4. Levels of IL-12 (A), TNF-α (B), and IL-6 (C) in the lungs isolated from test mice BALB/c mice were administered sublingually with *L. rhamnosus* (10^8 cfu/mouse) for 10 days before inoculation with the A/NWS/33 virus. Lung samples were collected and used for quantifying cytokines by ELISA on days 2, 4, and 6 p.i. The assay detection limit was 19.5 pg/mL for IL-12 and 15.625 pg/mL for TNF-α and IL-6. Statistically significance was determined by Student's *t*-test. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared with the results in the placebo group.

findings led us to investigate whether the sublingual route is useful for the delivery of probiotics against influenza virus infection. In this study, we demonstrated that the sublingual administration of *L. rhamnosus* provides significant protection against influenza

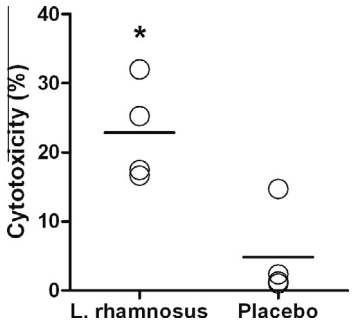

Fig. 5. Effect of sublingual administration of *L. rhamnosus* on the NK cell activity of splenocytes of influenza virus-infected mice BALB/c mice were administered sublingually with *L. rhamnosus* (10^8 cfu/mouse) for 10 days before inoculation with the A/NWS/33 virus. Splenic cells were prepared from the infected mice on day 4 p.i. for NK cell activity assay. Statistical significance was determined by Student's *t*-test. Asterisks indicate significant differences ($^*p < 0.05$) compared with the results in the placebo group.

virus infection in a mouse model. To the best of our knowledge, this is the first study to provide evidence that the sublingual administration of live lactobacilli can be effective in the prevention of influenza virus infection. We previously reported that the intranasal administration of live lactobacilli provides enhanced protection against influenza virus infection (Youn et al., 2012). However, safety issues remained, including aspiration pneumonia and abnormal immune responses such as a type I hypersensitivity responses due to bacteria taken up via an abnormal entry route. In contrast to intranasal administration, the sublingual route is considered to be more convenient and safer. Therefore, we speculate that the sublingual delivery of lactobacilli should not raise the same safety concerns as intranasal delivery, an issue that will be addressed in human trials.

One major advantage of the sublingual administration of *L. rhamnosus* is its ability to induce secretory IgA antibodies in the respiratory tract, the major target organ of influenza virus infection. Secretory IgA antibodies are considered major effectors in the adaptive immune defense of the respiratory mucosa (Underdown and Schiff, 1986). Although ingestion of lactobacilli protects against influenza virus infection by inducing serum IgG antibodies (Yasui et al., 1999) and IgA in bronchoalveolar lavage fluids (Kawashima et al., 2011; Salva et al., 2010), intranasally administered lactobacilli appears more effective for inducing protection against influenza virus infection, probably as a result of enhanced secretory IgA responses in the respiratory mucosa (Youn et al., 2012). In the present study, sublingual administration of *L. rhamnosus* also elicited high levels of anti-influenza virus-specific IgA in the lungs. Such secretory IgA antibody responses seem to play a role in preventing the entry and replication of the influenza virus in the respiratory tract, although the precise underlying mechanism remains unclear. Further research to investigate mechanisms is required.

As mentioned above, some probiotics have been shown to reduce the risk of viral infections such as the common cold and influenza (Hatakka et al., 2001; Leyer et al., 2009; Rautava et al., 2009). However, the mechanisms underlying the reductions in respiratory tract infections and other symptoms remains unclear. It is likely that these positive effects are due to the ability of probiotics to modulate immune stimulatory responses upon interaction with antigen-presenting cells such as dendritic cells (DCs) and macrophages. Several *in vitro* studies that show toll-like receptors (TLRs), especially TLR2, play a key role upon the stimulation of DC and macrophages with lactobacilli (Kawashima et al., 2011; Shida et al., 2009; Weiss et al., 2010; Zeuthen et al., 2008). 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 DC matured by microbial stimuli induces Th1 polarization and thus strongly stimulates of the adaptive immune defense. In the present study, we demonstrated that CD25 expression by CD^{4+} and CD^{8+} lymphocytes increased significantly in the lungs of mice in the *L. rhamnosus*-administered group compared to that in the naïve group at 6 days p.i. CD25, the α-chain of the IL-2 receptor, is a late activation marker induced during lymphoid cell activation and is expressed on activated T cells, B cells, and monocytes. Similar effects of other probiotic strains are reported in a few cases. For example, (Castellazzi et al., 2007) demonstrated that *L. paracasei* I 1688, *L. salivarius* I 1794, and a mixture of the two strains increase the percentages of CD^{4+}/CD^{25+} cells (T helper-activated regulatory T cells), CD^{8+}/CD^{25+} (T suppressor/cytotoxic-activated cells), and CD^{16+}/CD^{56+} (NK cells). Furthermore, *L. casei* Shirota also induced CD69 and CD25 expression in CD^{8+} and CD^{56+} subsets in human peripheral blood mononuclear cells *in vitro* (Dong et al., 2010). Thus, the present findings are concordant with these studies, indicating that probiotics enhance the activation of helper and cytotoxic T lymphocytes and beneficially modulate both innate and adaptive immunity.

Th1 and antiviral cytokine production as well as NK cell activity are important for preventing influenza infection. NK cells are a major component of host-nonspecific cell-mediated immunity, and recognize and help to control a wide range of pathogens including viruses, bacteria, and intracellular parasites (Peakman and Vergani, 1997). Several studies indicate that lactobacilli administration can characteristically activate NK cells and thus protect the host against influenza virus infection (Harata et al., 2010; Hori et al., 2001, 2002; Kawashima et al., 2011; Yasui et al., 2004, 1999). To determine the underlying mechanism by which sublingual administration of *L. rhamnosus* protects against influenza virus infection in mice, NK activity in the spleen and the related cytokines in the lungs were measured. In the present study, IL-12 production was augmented by the sublingual administration of *L. rhamnosus* in the lungs from mice on day 2 p.i. IL-12 is an important Th1 cytokine for cellular immunity (Kobayashi et al., 1989) that enhances IFN-γ production from NK cells and stimulates cytotoxic T and NK cells (Gately et al., 1994). The increase in IL-12 following sublingual administration of *L. rhamnosus* on day 2 p.i. suggests NK cells may be activated in the lungs of the infected mice. NK cell activity in mice elicited by influenza infection is increased in the lung as well as in the spleen (Culley, 2009; Ritz et al., 2006; Stein-Streilein et al., 1983). Concordantly, we demonstrated that NK activity in the splenic cells of infected mice on day 4 p.i. was significantly higher in the *L. rhamnosus*-treated group than that in the placebo group. It is likely that the sublingual administration of *L. rhamnosus* activated NK cells via the augmentation of IL-12 production elicited by influenza infection in mice.

Because of the pronounced elevation of cytokines during H5N1 infection, cytokine storm is widely hypothesized to be the main cause of pathology, which ultimately leading to death. In particular, elevated levels of proinflammatory cytokines including TNF-α, IL-6, and CC chemokine ligand 2 (CCL2), have been detected in human cells and mice infected with highly pathogenic H5N1 influenza virus (Chan et al., 2005; Cheung et al., 2002; de Jong et al., 2005; Lee et al., 2005; Szretter et al., 2007; Xu et al., 2006). In the present study, high levels of the proinflammatory cytokines IL-6 and TNF-α were also detected in the lungs of influenza-infected mice. However, the elevate IL-6 and TNF-α levels in the lungs of infected mice were significantly suppressed by the sublingual administration of *L. rhamnosus*. Considering that IL-6 and TNF-α levels positively correlate with lung inflammation and vascular dysfunction, these results suggest that the decreased levels of pro-inflammatory cytokine might also contribute to the protection against influenza virus.

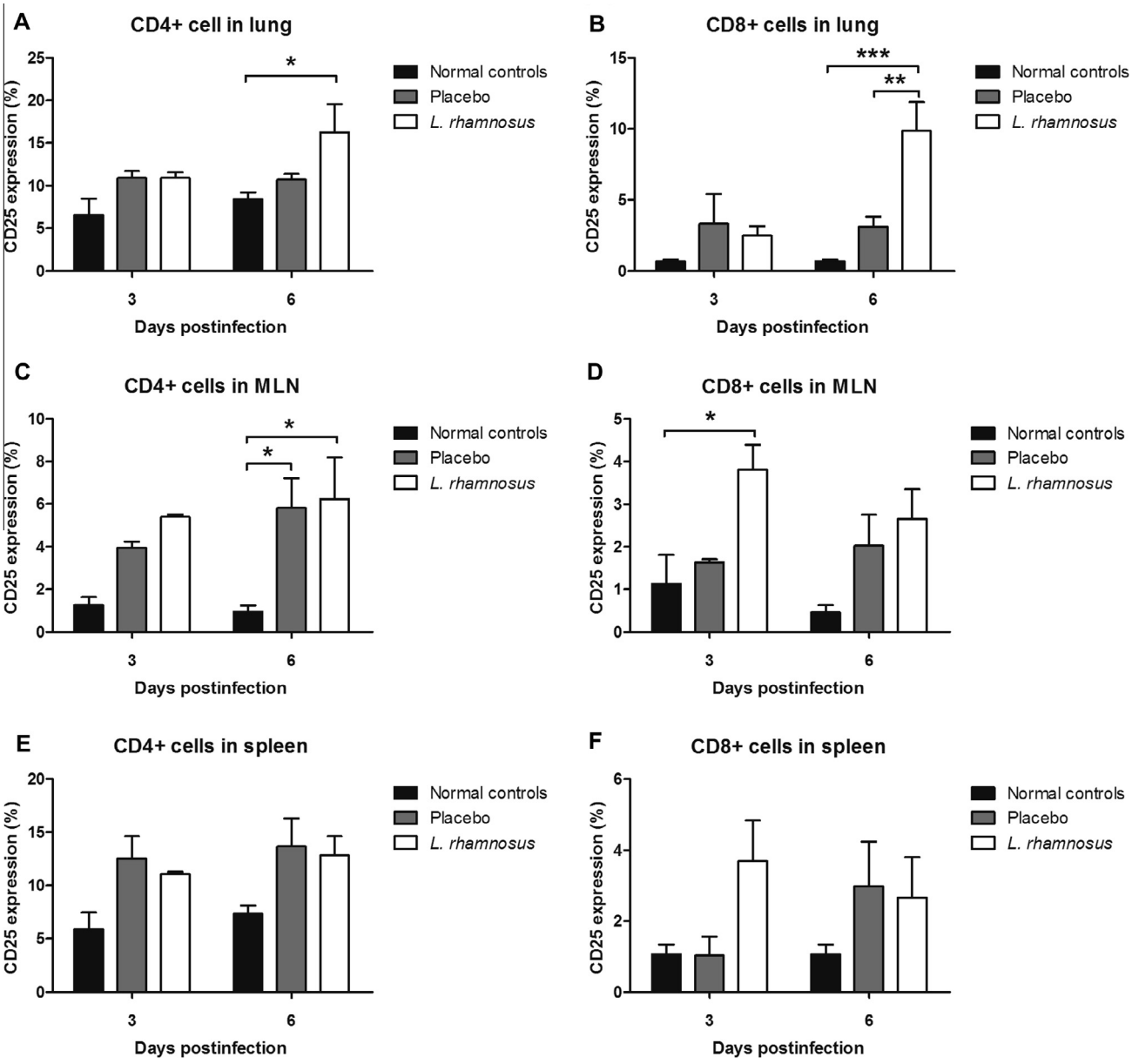

Fig. 6. Effect of sublingual administration of *L. rhamnosus* on CD25 expression by lymphocytes in the lungs, mediastinal lymph nodes (MLNs), and spleen of influenza virus-infected mice BALB/c mice were administered sublingually with *L. rhamnosus* (10^8 cfu/mouse) for 10 days before inoculation with the A/NWS/33 virus. Lymphocytes in the lungs (A and B), MLNs (C and D), and spleen (E and F) were prepared from the infected mice on days 3 and 6 p.i. to evaluate T cell activation. Statistical significance was determined by 2-way ANOVA. Asterisks indicate significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) compared with the results in the naïve or placebo group.

In conclusion, sublingual administration of *L. rhamnosus* provides enhanced protection against influenza virus infection by enhancing of mucosal secretory IgA production and T cell and NK cell activity. Many issues regarding sublingual administration remain unresolved, including the improvement of formulations that would enable enhanced efficacy and lowered required dose. Nonetheless, our findings strongly suggest that compared to traditional application, sublingual delivery could be a more effective avenue for probiotics against seasonal and influenza as well as the next pandemic influenza.

Acknowledgements

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