Chapter 5

Efficacy of oral administration of heat-killed probiotics from Mongolian dairy products against influenza infection in mice: Alleviation of influenza infection by its immunomodulatory activity through intestinal immunity

5.1 Introduction

Influenza virus (IFV) infects the respiratory tract in humans and causes a variety of symptoms including fever, nasal secretions, cough, headache, muscle pain, and pneumonia. These clinical symptoms often become severe, especially in high-risk groups such as the elderly and infants, who may be immunologically incompetent (Nicholson *et al*. 2000; Thompson *et al*. 2003). In IFV infection, cytokines are produced locally and systemically, and their production is important in promoting the host-immune defense to alleviate symptoms, especially in the early stage of infection (Van Reeth 2000; Kaiser *et al*. 2001; Kash *et al*. 2006). In a murine IFV-infection model, cytokines such as interleukin (IL)-12 and -18 as T-helper (Th) 1 immune-response mediators, interferon (IFN)-γ as a Th1 cytokine, IL-4 and -10 as Th2 cytokines, IL-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α as proinflammatory cytokines, and IFN-α and -β have been shown to be produced in the respiratory tract (Hennet *et al*. 1992; Kurokawa *et al*. 1996; Tsurita *et al*. 2001; Kurokawa *et al*. 2002, 2010). Recently proinflammatory cytokines have been reported to be markedly elevated in human cells and mice during infection with the highly pathogenic H5N1 IFV (Cheung *et al*. 2002; Xu *et al*. 2006; Szretter *et al*. 2007). The occurrence of the 'cytokine storm' has been proposed to contribute to the increased severity of the diseases

caused by this highly pathogenic virus (Yuen *et al*. 1998; Peiris *et al*. 2004; de Jong *et al*. 2006). Host immunity including cytokine production has been suggested to contribute to influenza symptoms.

Some nutritional and natural components have been studied for their effectiveness in treating IFV infection by controlling production of cytokines (Cui *et al*. 2000; Kurokawa *et al*. 2002; Shin *et al*. 2005; Ritz *et al*. 2006: Kurokawa *et al*. 2010). Lactic acid bacteria (LAB) have been used worldwide in many kinds of foods, especially dairy products that are mainly taken orally. Some LABs are called probiotics, which are living microorganisms that confer a health benefit on the host when administered in adequate amounts (World Health Organization 2001). It was reported that some probiotics have potential as immunomodulators and modulated the symptoms of diseases. For example, the probiotic LAB *Lactobacillus* (*L*.) *paracasei* KW3110 strain was reported to improve the Th1/Th2 imbalance by inducing IL-12 in Th2-polarized murine splenocytes *in vitro*, and oral administration of KW3110 in a mouse allergy model directed the Th1/Th2 balance toward Th1 through the maturation of antigen-presenting cells and inhibition of serum immunoglobulin E elevation (Fujiwara *et al*. 2004). Moreover, it was shown that the KW3110 strain was effective on atopic dermatitis-like skin lesions in mice and cedar pollinosis in Japanese (Wakabayashi *et al*. 2008; Yonekura *et al*. 2009). Thus, LABs are possible immunomodulators and may be expected to play an important role in the pathogenesis of IFV infection by modulating host immune responses.

Previously, 10 LAB strains from traditional Mongolian dairy products were isolated and characterized them as probiotics *in vitro* (Takeda *et al*. 2011b). In this study, to evaluate the immunomodulatory activity of the 10 LABs in IFV infection, I assessed their protective effects on the host-defensive immunity including cytokine production in a murine model of IFV infection. Among the 10 LAB strains, the *L. plantarum* 06CC2 strain was found to be effective against IFV infection in mice, and its immunomodulatory activity through

intestinal immunity was evaluated in relation to the alleviation of IFV infection.

5.2 Materials and methods

5.2.1 Viruses and cells

IFV A/PR/8/34(H1N1) was provided by H. Ochiai, Toyama University, Toyama, Japan (Sawamura *et al*. 2010). IFV were propagated in Madin-Darby canine kidney (MDCK) cells, and virus titers in the stock solution were determined by a plaque assay (Shimizu *et al*. 2008). MDCK cells were grown and maintained in Eagle's minimum essential medium supplemented with 2% and 5% heat-inactivated fetal bovine serum, respectively. YAC-1 cells (RIKEN, Ibaraki, Japan) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and used for a natural killer (NK) cell activity assay.

5.2.2 Lactic acid bacteria

Ten LAB strains with potential as probiotics were isolated from Mongolian dairy products (Table 5-1). The LABs were cultured at 37˚C for 24 h in MRS (Man, Rogosa and Sharpe) broth (Merck, Darmstadt, Germany) as described previously (Takeda *et al*. 2011b). They were harvested by centrifugation at 10,000×g for 5 min, washed twice with phosphate-buffered saline (PBS), and boiled for 1 h. Then, the boiled LABs were washed again with PBS and lyophilized. The lyophilized powder was suspended in distilled water for oral administration to mice.

Table 5-1 Species and origin of LAB strains

Strain	Origin
L. plantarum 05AM23	Alcoholic fermented mare milk (Airag)[a]
L. plantarum 06TCa8	Fermented camel milk (Tarag)[b]
L. paracasei ssp. *paracasei* 06TCa19	Fermented camel milk (Tarag)
L. paracasei ssp. *paracasei* 06TCa22	Fermented camel milk (Tarag)
L. paracasei ssp. *tolerans* 06TCa39	Fermented camel milk (Tarag)
L. plantarum 06TCa40	Fermented camel milk (Tarag)
L. paracasei ssp. *paracasei* 06TCa43	Fermented camel milk (Tarag)
L. plantarum 06CC2	Cow milk cheese (Aaruul)[C]
L. delbrueckii ssp. *lactis* 06TC3	Fermented cow milk (Tarag)
L. plantarum 06CC9	Cow milk cheese (Aaruul)

[a] Airag is fermented horse milk.

[b] Tarag is a traditional Mongolian yoghurt.

[C] Aaruul is a traditional Mongolian hard cheese.

5.2.3 Mice

Specific-pathogen-free female BALB/c mice (6-week-old, 17–19 g) were obtained from Kyudo Animal Laboratory, Kumamoto, Japan. The mice were housed five per cage in specific pathogen-free conditions under a 12 h light/dark cycle. The temperature in the room was kept at 23 ± 2˚C. They were fed a standard pellet diet CE-2 (Clea Japan, Inc., Tokyo, Japan) and water *ad libitum*. The mice were acclimated for 5 to 7 days before starting the experiments. The experimental protocols were approved by the Animal Experiment Committee of Kyushu University of Health and Welfare, Japan, and the animal experimentation guidelines of the university were followed in the animal studies (Permission No. 23-1-05).

5.2.4 Influenza virus infection in mice

Ten LAB strains were examined for their efficacy against IFV infection in mice. Mice were intranasally infected or mock-infected with 500 plaque-forming units (PFU) of A/PR/8/34 strain in 20 μl of PBS under anesthesia with intramuscular ketamine and xylazine at 42 and 7.6 μg/g of body weight, respectively, as described by Sawamura *et al*. (2010). Lyophilized LAB powder was suspended in distilled water at a concentration of 10% (w/v) and the suspension of 0.2 ml per mouse was orally administered by gavage to mice twice daily. In this experiment, I used 20 mg/mouse of LAB as the maximum dose for mice to evaluate the potential for immunomodulatory activity. The dose was deduced from the maximum dose of supplemental foods, such as yoghurt, for humans and corresponded to a maximum level to avoid diarrhea in mice. As a control, mice were given an equal volume of distilled water. Oral administration began 2 days before infection and continued until 7 days after infection. The body weights and life and death of the mice were monitored daily.

5.2.5 Infiltrated cells

The effect of oral administration of *L. plantarum* 06CC2 strain on the number of infiltrated cells in bronchoalveolar lavage fluid (BALF) prepared from the lungs of infected mice was examined. BALF was prepared on days 2, 4, and 6 after infection as described previously (Tsurita *et al*. 2001; Shimizu *et al*. 2008). The BALF was centrifuged at 1,000 rpm for 10 min at 4˚C, and the cells and supernatants were separately collected. The infiltrated cells in the BALF were dispersed in PBS and stained with trypan blue, and the total number was counted. BALF was also smeared on glass slides and stained with Diff-Quick (Sysmex, Hyogo, Japan). Then the numbers of macrophages, neutrophils and lymphocytes in BALF were histopathologically determined and counted under a microscope at Sapporo General Pathology Laboratory, Hokkaido, Japan. The supernatant was stored at -80˚C until cytokine assay.

5.2.6 Virus yields in lungs

The effect of oral administration of 06CC2 strain on virus yields in the lungs of IFV-infected mice was examined. Lungs were removed under anesthesia on days 2, 4, and 6 after infection and homogenized in 2 ml of PBS on ice. The homogenate was centrifuged at 3,000 rpm for 15 min, and the virus yield in the supernatant was determined by the plaque assay on MDCK cells (Shimizu *et al*. 2008; Kurokawa *et al*. 2010). Briefly, confluent monolayers of MDCK cells were incubated with the supernatant of BALF serially diluted in PBS containing 1% bovine serum albumin for 1 h at room temperature. Then the cells were overlaid with nutrient agarose (0.8%) medium and cultured at 37˚C for 3 days (Sawamura *et al*. 2010). The cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution. Visualized plaques were counted under a dissecting microscope.

5.2.7 Cytokine assay

Cytokine levels in the BALF from mock-infected and infected mice were determined using ELISA kits for mouse IFN-γ, IL-12, TNF-α, IL-6, IFN-α, and IFN-β according to the manufacturer's instructions (eBioscience, San Diego, CA, USA or R&D Systems, Minneapolis, MN, USA).

5.2.8 Natural killer cell activity in spleen cells

The effect of oral administration of the *L. plantarum* 06CC2 strain on splenic NK cell activity in IFV-infected mice was examined by using a cytotoxicity assay kit (CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega Corporation, 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. Spleen cells as effector cells were prepared from infected mice and erythrocytes were depleted from the spleen cells by treatment with 0.144 M NH_4Cl in 0.017 M Tris-HCl buffer (pH 7.65). The effector ($5 \times 10^5 – 5 \times 10^4$ cells/well) and target cells (1×10^4 cells/well) were mixed at a ratio of 50:1, 10:1, or 5:1, 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 collected 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 mesured at 490 nm by a microplate reader (Bio-rad laboratories Corporation, Tokyo, Japan). The percentage of specific release was calculated according to the following equation:

Specific lytic activity (%)=[(A_{490} of effector and target cell mix – A_{490} of spontaneous effector LDH release – A_{490} of spontaneous target LDH release) / (A_{490} of maximum target LDH release – A_{490} of spontaneous target LDH release)] × 100

5.2.9 Gene expression in Peyer's patch

The mRNA expression of Toll-like receptors (TLRs) 2, 3, and 4, IFN-γ, and IL-12 receptor β2 in Peyer's patches was examined by real time reverse transcriptase - PCR with a Roche LightCycler P2000 real-time PCR system (Roche Diagnostics, Indianapolis, IN), and the data were evaluated by normalizing results to those of mouse β-actin. Mice (3 to 6 mice in a group) were infected and mock-infected with the influenza virus and administered with or without 06CC2 strain as described above. On day 2 after infection, 6 to 5 Peyer's patches were freshly prepared from the small intestine of each mouse in a group and the cells prepared from the all fresh Peyer's patches removed from a mouse were gathered. RNA was isolated from the gathered cells using trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The isolated RNA was transcribed into cDNA by ReverTra Ace α (Toyobo Co. Ltd, Osaka, Japan) using an oligo-dT(20) primer according to the manufacturer's instructions. For the TLR 2, I used the method of Yoshida *et al*. (2010). Briefly, PCR amplification was performed by using LightCycler FastStart DNA Master Plus SYBR Green I kit (Roche Diagnostics) with the TLR 2 specific primers (forward: 5'-TCCCTTGACATCAGCAGGAACACT-3', reverse: 5'-ACTAACATCCAACACCTCCAGCGT-3') and the β-actin primers (forward: 5'-AGCCATGTACGTAGCCATCC-3', reverse: 5'-TCCCTCTCAGCTGTGGTGGTGAA-3'). Then, samples were measured in the real time PCR system for an initial denaturation at 95˚C for 10 min, followed by 40 PCR amplification cycles of 95˚C for 10 s, 60˚C for 5 s, and 72˚C for 15 s, and the relative quantity of gene was determined by using the comparative C_t method and normalized by β-actin. For the TLRs 3 and 4, IFN-γ, and IL-12 receptor β2, the method of Watanabe *et al*. (2010) was used. Briefly, amplification and analysis of the cDNA of TLR 3 were performed by the real time PCR system using LightCycler FastStart DNA Master Plus SYBR Green I kit with the TLR 3

specific primers (forward: 5’-GATACAGGGATTGCACCCATA-3’, reverse: 5’-TCCCCCAAAGGAGTACATTAGA-3’). Then, samples were measured in the real time PCR system for an initial denaturation at 95˚C for 10 min, followed by 45 PCR amplification cycles of 95˚C for 10 s, 60˚C for 5 s, and 72˚C for 10 s. The amounts of cDNA (numbers of copies) of TLR 3 was determined by comparing the crossing point values of the cDNA samples to those of the TA vectors harboring parts of the murine genes of TLR 3 (nt. 148-480). The amounts of TLR 4, IFN-γ, and IL-12 receptor β2 cDNA were amplified and analyzed on the real time PCR system using a LightCycler FastStart DNA Master HybProbe kit (Roche Diagnostics) with a mouse TLR 4, a mouse IFN-γ, and a mouse IL-12 receptor β2 primer-probe kit, respectively (Nihon Gene Research Laboratories, Inc., Sendai, Japan) according to the manufacturer’s instructions. The amounts of TLR 4, IFN-γ, and IL-12 receptor β2 cDNAs (number of copies) were determined according to the manufacturer’s instructions. Amplification and analysis of the β-actin cDNA were performed using a Roche LightCycler FastStart DNA Master SYBR Green I kit with a pair of β-actin specific primers (forward: 5’-TGGAATCCTGTGGCATCCATGAAAC-3’, reverse: 5’-TAAAACGCAGCTCAGTAACAGTCCG-3’). The amounts of β-actin cDNA were determined also by comparing the crossing point value of cDNA sample to those of plasmid, a TA vector harboring part of the murine β-actin gene (nt. 728-1076).

5.2.10 Statistical analysis

Repeated measures two-way analysis of variance (repeated measures ANOVA) was used to analyze the interaction between LAB and water in the net body weight changes of infected mice from day 0 to day 6 after infection and virus yields in lungs for 2 to 6 days after infection. Student’s *t*-test was used to evaluate the statistical significance of differences between two groups in the numbers of infiltrated cells, concentrations of cytokines, percent

cytotoxicities, and amounts of gene expression. Statistical differences in the mortality of mice were evaluated using the Kaplan-Meier method with a log-rank test. A *P*-value of less than 0.05 was defined as statistically significant.

5.3 Results

5.3.1 Efficacy of lactic acid bacteria on influenza infection in mice

An intranasal influenza virus infection model in mice was used to screen the potential activity of LAB as an experimental immunomodulator. This model is a lethal model with pneumonia caused by host immune response against influenza virus infection (Tashiro *et al* 1987a, b). However, in this infection model, not only lethality but also the severity of influenza symptoms associated with the development of typical pneumonia with pathologic changes similar to those in humans can be evaluated by the body weight loss of infected mice (Kurokawa *et al*. 1996; Tsurita *et al*. 2001; Kurokawa *et al*. 2002, 2010). Thus, in the screening, 10 LAB strains were examined for their protective effects of body weight loss of IFV-infected mice (n = 5) to select the candidates of LAB strains that can alleviate influenza symptoms (Fig. 5-1A). In this murine influenza virus infection model, oral administration of oseltamivir at 1 mg/kg twice daily for days 0 to 4 after infection was significantly effective in reducing the body weight loss of infected mice and virus titers in the BALF (Sawamura *et al*. 2010). In this study, I used 20 mg/mouse of LAB as a maximum dose for mice to assess the potential of LAB as a supplemental food assumed to be an immunomodulator and LAB was administered to IFV-infected mice twice daily from 2 days before infection. As shown in Figure 5-1A, the mean body weights of water-administered mice decreased markedly after infection. However, all LAB strains administered were effective in protecting the mean body weight loss, and the 06CC2 and 06TC3 strains especially were significantly effective ($P <$

0.01 by repeated measures ANOVA from day 0 to day 6). Further, I evaluated whether the protection of body weight loss by 06CC2 and 06TC3 strains correlate with the delay of survival time of infected mice and/or the decrease of lethality. As shown in Figure 5-1B, the 06CC2 strain was significantly effective in protecting the body weight loss of infected mice (n = 10, $P < 0.01$ by repeated measures ANOVA from day 0 to day 6), although the protection of body weight loss seemed to be moderate as compared with that in the first screening shown in Figure 5-1A. The significant protection of body weight loss of infected mice by the 06CC2 strain was confirmed. Moreover, the 06CC2 strain was significantly effective in prolonging the survival times ($P < 0.05$ by the Kaplan-Meier method from day 0 to day 14) (Fig. 5-1C). However, the 06TC3 strain failed to prolong the survival time, although it was significantly effective in protecting the body weight loss (data not shown). There was no significant difference between the mean changes of body weights of mock-infected mice (n=7) administered water and the 06CC2 strain at 20 mg/mouse at 10 days after infection (19.8 ± 0.5 and 19.4 ± 1.1 g, respectively), and no death was observed. Thus, the 06CC2 strain was significantly effective in protecting the body weight loss of IFV-infected mice and prolonging the survival time without toxicity. I selected 06CC2 strain as a candidate of immunomodulator that can alleviate influenza infection.

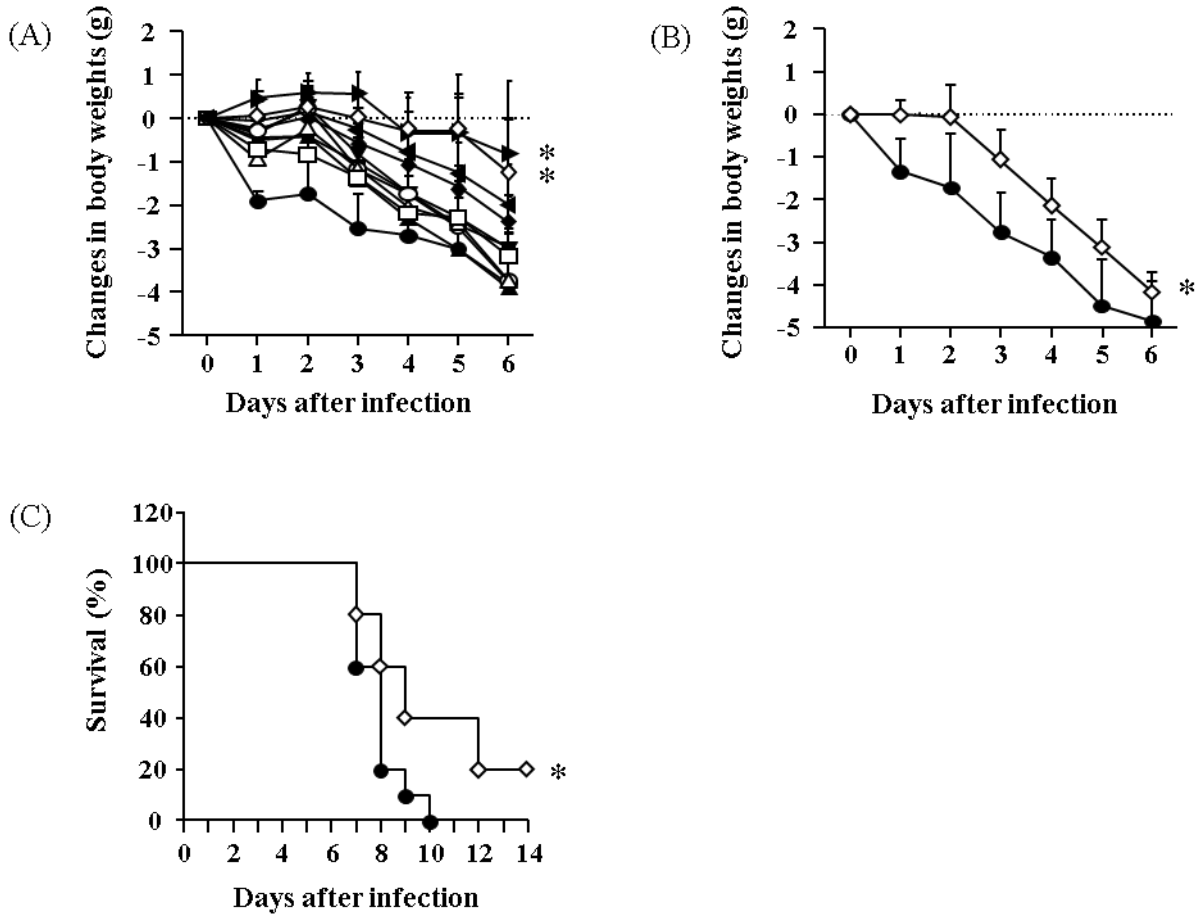

Figure 5-1 Effects of LAB on IFV infection in BALB/c mice.

In each experiment, mice were infected with 500 pfu of IFV A/PR/8/34 intranasally and the LAB oral administration began 2 days before infection and continued until 7 days after infection. The bars indicate standard deviation. (A) Changes in the mean body weights of IFV-infected mice (5 mice per group). LAB 05AM23 (▲), 06TCa8 (■), 06TCa19 (▼), 06TCa22 (♦), 06TCa39 (○), 06TCa40 (Δ), 06TCa43 (□), 06CC2 (◊), 06TC3 (►), and 06CC9 (◄) strains (20 mg/mouse) and water (●) were orally administered to the infected mice twice dairy. The asterisks indicate the weight changes in groups administered strains 06CC2 and 06TC3 for 0–6 days and the statistical significances versus infected mice administered water for days 0–6 after infection (*P < 0.05) by repeated measures ANOVA. (B) Changes in the mean body weights of IFV-infected mice (10 mice per group). LAB 06CC2 (◊) strain at 20 mg/mouse and water (●) were orally administered to the infected mice twice dairy. The asterisk indicates the body weight changes in the group administered 06CC2 for 0–6 days after infection (*P < 0.05) by repeated measures ANOVA. (C) Effect of 06CC2 strain on the survival times of IFV-infected mice (10 mice per group). LAB 06CC2 (◊) strain at 20 mg/mouse and water (●) were orally administered to the infected mice twice dairy. The asterisk indicates that a significant difference was between the group administered the 06CC2 strain and the water-administered group (*P < 0.05) by the Kaplan-Meier method for 0–14 days after infection.

5.3.2 Effect of 06CC2 strain on virus yeilds in lungs

The effect of the 06CC2 strain on virus yields in lungs was examined on days 2, 4, and 6 after IFV infection, as shown in Figure 5-2. Significant differences of virus yields in lungs on days 2, 4 and 6 were observed between 06CC2 administration and water administration (control) ($P < 0.01$ by repeated measures ANOVA from day 2 to day 6). The 06CC2 stain was significantly effective in reducing virus yields in the lungs of infected mice.

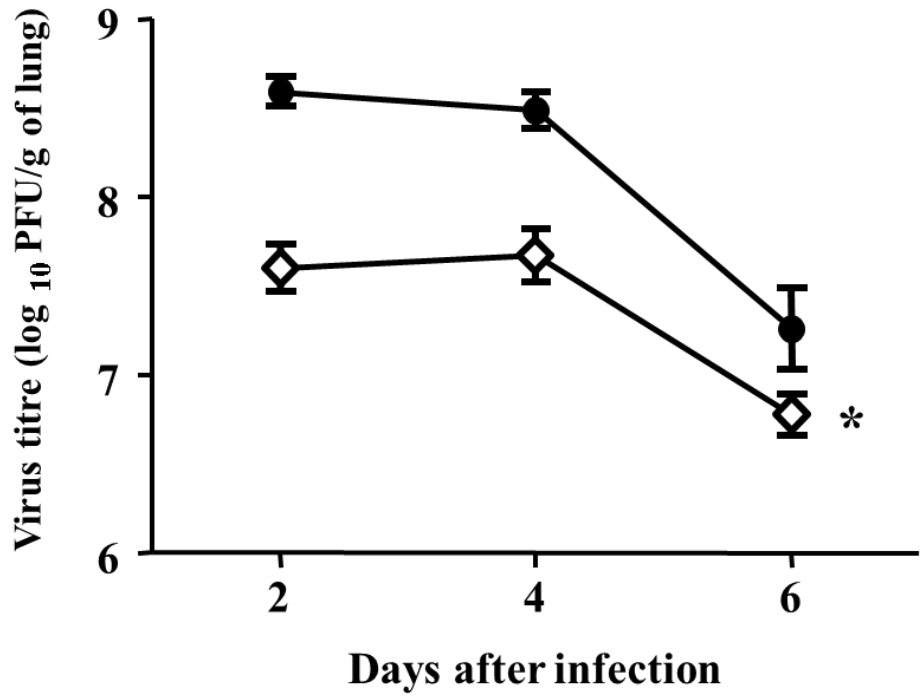

Figure 5-2 Effect of 06CC2 strain on virus yields in the lungs of IFV infected mice.

Mice were intranasally infected with IFV, and water (●) or 06CC2 strain (◊) was orally administered twice daily from 2 days before infection. Lungs were removed under anesthesia on days 2, 4, and 6 after infection and homogenized in 2 ml of PBS. The virus yields in the supernatants of homogenates were determined by the plaque assay on MDCK cells. Six mice were used in each group. Bars indicate standard deviation. The asterisk indicates that the difference is statistically significant compared to the water-administered group (*$P < 0.01$) by repeated measures ANOVA for days 2 to 6.

5.3.3 Effects of 06CC2 strain on cytokine production in bronchoalveolar lavage fluid

To evaluate the potential activity of the 06CC2 strain as an immunomodulator in influenza infection, its effects on cytokine levels produced by influenza infection were examined in IFV-infected mice. I examined the levels of Th1 cytokines (IFN-γ and IL-12), IFN-α and β, and proinflammatory cytokines (TNF-α and IL-6) in the BALF from infected mice on days 2, 4, and 6 after infection (Fig. 5-3A-E). In infected mice both with and without 06CC2 administration, the levels of IL-12 (Fig. 5-3B), TNF-α (Fig. 5-3C), and IFN-α (Fig. 5-3E) on day 2 were higher than those on days 4 and 6, while the levels of IFN-γ (Fig. 5-3A) and IL-6 (Fig. 5-3D) increased markedly on day 6 after infection. On day 2, the levels of IFN-γ, IL-12, and IFN-α in infected mice administered the 06CC2 strain were significantly higher than those in the controls ($P < 0.05$ by Student's *t*-test), but the level of TNF-α was significantly lower than that in the control mice ($P < 0.05$ by Student's *t*-test). On day 6, 06CC2 administration significantly reduced the level of IL-6 ($P < 0.05$ by Student's *t*-test). The level of IFN-γ was also reduced by 06CC2 administration, although it was not statistically significant. The level of IFN-β was lower than that of IFN-α from days 2 to 6, and 06CC2 administration had no effect on the levels of IFN-β in the serum and BALF of infected mice (data not shown). In the BALF from mock infected mice, the levels of IL-12, IFN-γ, and TNF-α were undetectable levels (data not shown). Thus, oral administration of the 06CC2 strain was effective in elevating the production of IFN-α and Th1 cytokines (IL-12 and IFN-γ) and suppressing TNF-α production in the early phase of infection and IL-6 production in the later phase of infection.

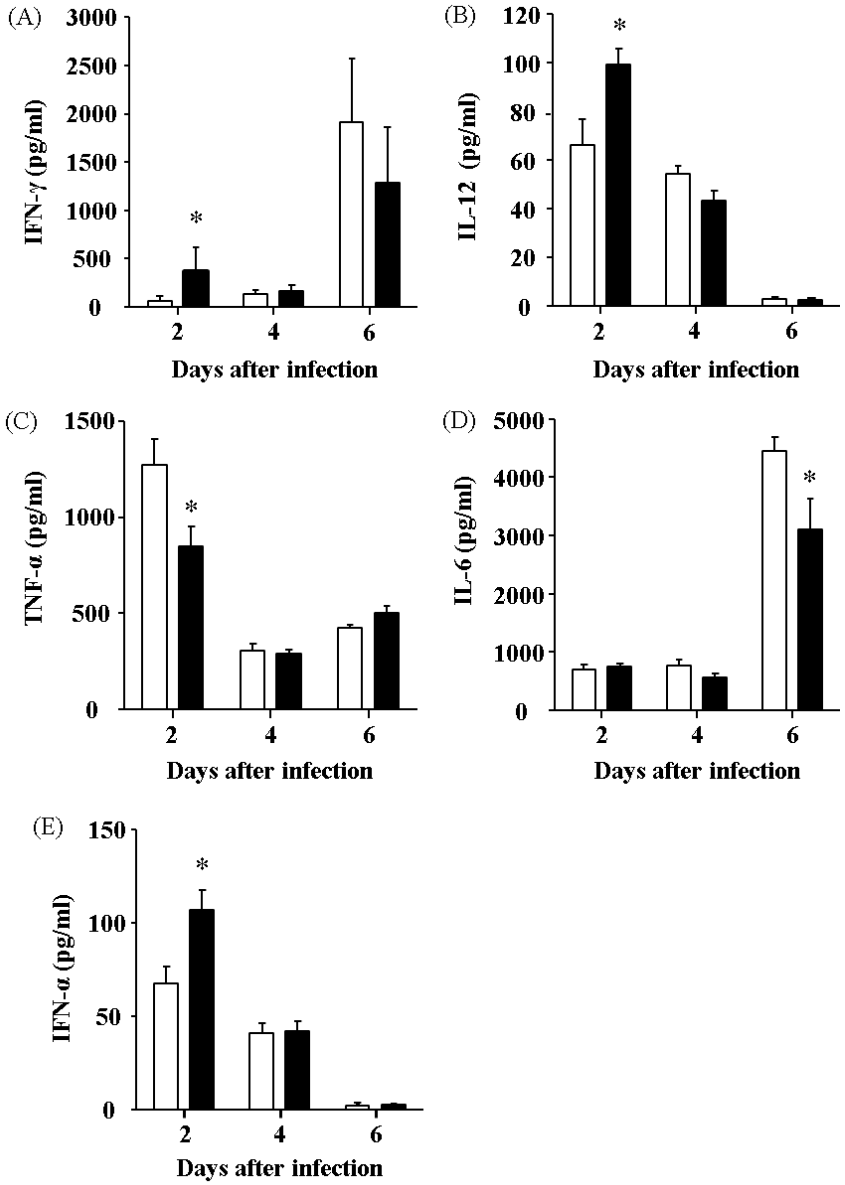

Figure 5-3 Effects of 06CC2 strain on cytokines level in BALF of IFV infected mice.

Changes of IFN-γ (A), IL-12 (B), TNF-α (C), IL-6 (D), and IFN-α (E) levels in BALF of IFV-infected mice administered the 06CC2 strain. Water (open columns) or 06CC2 strain (closed columns) were administered to the infected mice, and BALF was prepared from the mice on days 2 (n = 5), 4 (n = 5), and 6 (n = 6) after infection. The levels of IFN-γ, IL-12, TNF-α, IL-6, and IFN-α in the BALF were determined by ELISA. Bars indicate standard deviation. The asterisks indicate statistical significance on each day (*$P < 0.05$) by Student's-t test.

5.3.4 Effects of 06CC2 strain on numbers of infiltrated cells in bronchoalveolar lavage fluid

The numbers of total infiltrated cells, macrophages, neutrophils, and lymphocytes in the BALF prepared from infected mice administered the 06CC2 strain or water (control) were compared on days 2, 4, and 6 based on a histopathological examination (Table 5-2). The numbers of total infiltrated cells, macrophages, neutrophils, and lymphocytes examined in both groups increased in a time-dependent manner as reported previously (Hashiba *et al*. 2001; Shin *et al*. 2005). On day 2, the numbers of total infiltrated cells, macrophages, and neutrophils in BALF from infected mice administered 06CC2 were significantly lower than those in the control mice ($P < 0.05$ by Student's *t*-test), but no significant differences were observed on days 4 and 6. Oral administration of the 06CC2 strain did not affect the numbers of lymphocytes throughout days 2 to 6. The oral administration of the 06CC2 strain was effective in suppressing the numbers of total infiltrated cells, especially macrophages and neutrophils, in the BALF of infected mice on day 2.

Table 5-2 Numbers of infiltrated cells in BALF of IFV-infected mice

Cells ($\times 10^4$ cells/ml)	Water			06CC2		
	Day 2	Day 4	Day 6	Day 2	Day 4	Day 6
Total cells	78.8 ± 5.9	546.0 ± 185.1	1460.0 ± 194.9	52.2 ± 19.4*	680.0 ± 60.4	1240.0 ± 114.0
Macrophages	18.2 ± 4.8	186.0 ± 53.2	640.0 ± 149.8	9.9 ± 3.5*	248.0 ± 28.6	508.0 ± 78.6
Neutrophils	59.0 ± 6.2	328.0 ± 127.6	766.0 ± 114.1	40.4 ± 14.7*	404.0 ± 38.5	662.0 ± 61.8
Lymphocytes	1.5 ± 1.0	32.0 ± 1.8	56.2 ± 32.1	1.9 ± 2.0	27.0 ± 1.2	70.0 ± 23.6

* $P < 0.05$ when compared with the numbers of cells of the water group on each day by Student's *t*-test.

The numbers were shown mean ± SD.

The numbers of infiltrated cells in the BALF of five mice were counted.

5.3.5 Effect of 06CC2 strain on NK cell activity in spleen cells

NK cells play an important role as the first defense against IFV infection in the early phase, and NK cell activity was elevated not only in lungs but also in spleens of infected mice (Stein-Steilein *et al*. 1983; Ritz *et al*. 2006; Culley 2009). I examined the effect of 06CC2 administration on NK cell activity in spleen cells of infected mice on days 2 and 4 after infection (Figs. 5-4A and 5-4B, respectively). NK cell activity in the spleen cells of IFV-infected mice was elicited in a ratio-dependent manner of effector/target cells on days 2 and 4. The NK cell activity was significantly enhanced by 06CC2 administration at 50:1 on day 2 ($P < 0.05$ by Student's *t*-test) but not at 10:1 and 5:1. On day 4, no significant elevation of NK cell activity was observed at any ratio. Thus, 06CC2 administration was significantly effective in enhancing NK cell activity on day 2 after infection.

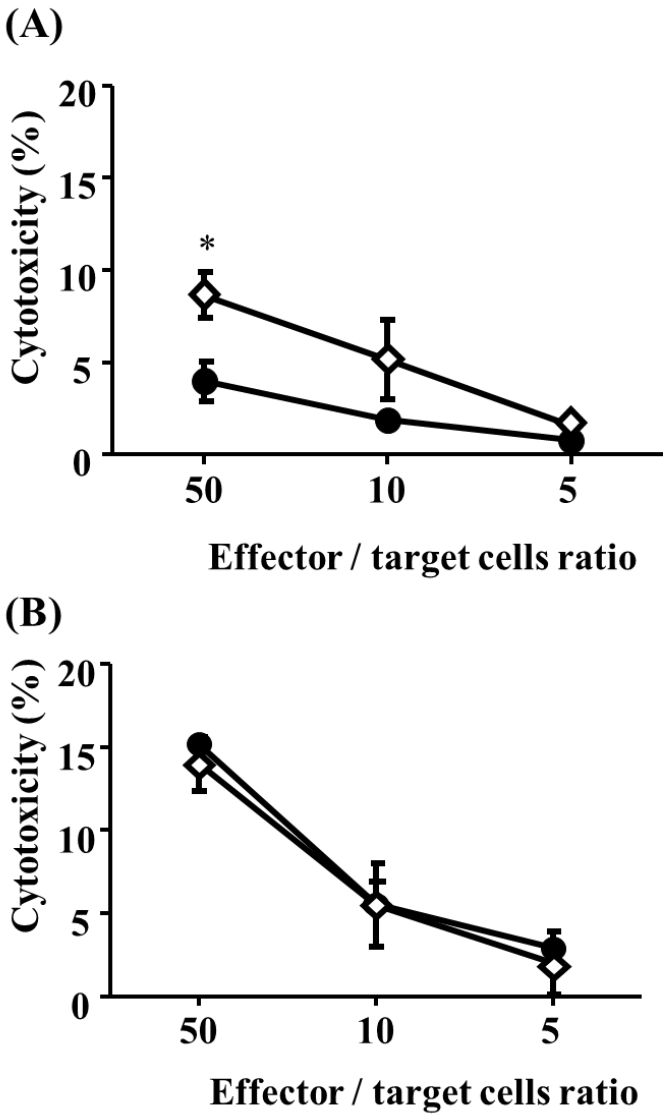

Figure 5-4 Effect of 06CC2 strain on NK cell activity in spleen cells of IFV infected mice.

Water (●) or 06CC2 strain (◊) was orally administered to IFV-infected mice, and spleen cells were prepared from infected mice on day 2 (A) and day 4 (B) for NK cell activity assay. Four mice were used in each group. Bars indicate standard deviation. The asterisk indicates statistical significance versus the water-administrated group at each ratio by Student's-t test. (*$P < 0.01$)

5.3.6 Effect of 06CC2 strain on mRNA expressions of IL-12 receptor, IFN-γ and TLRs in Peyer's patches

Peyer's patches are an intestinal immune organ, where the 06CC2 strain may first give an influence on host immune system. Oral administration of LAB to mice has been reported to augment IL-12 and IFN-γ mRNA expression in Peyer's patches (Tsai *et al*. 2010). TLRs are known as a family of receptors that play crucial roles in innate immune system. Because the 06CC2 strain significantly augmented the levels of IFN-γ and IL-12 in BALF and NK activity in spleen on day 2 after infection, I examined the effect of 06CC2 strain on the gene expressions of IFN-γ, IL-12 receptor β2, and TLRs 2, 3, and 4 in Peyer's patches of infected mice on day 2 (Fig. 5-5). The administration of 06CC2 strain significantly augmented the mRNA expression of IFN-γ in Peyer's patches compared with the control ($P < 0.05$ by Student's *t*-test). The mRNA expression of IL-12 receptor β2 was also elevated, although the significant difference was not observed ($P = 0.0519$ by Student's *t*-test). However, IL-12 receptor and IFN-γ mRNA expression in the Peyer's patches of mock-infected mice was not affected by 06CC2 administration (data not shown). Also, 06CC2 administration had no effect on the mRNA expressions of TLRs 2, 3, and 4 in the Peyer's patches of infected mice. Thus, the mRNA expression of IFN-γ and IL-12 receptor β2 was augmented in the Peyer's patches of infected mice on day 2 after infection by 06CC2 administration, but those of TLR 2, 3, and 4 were not.

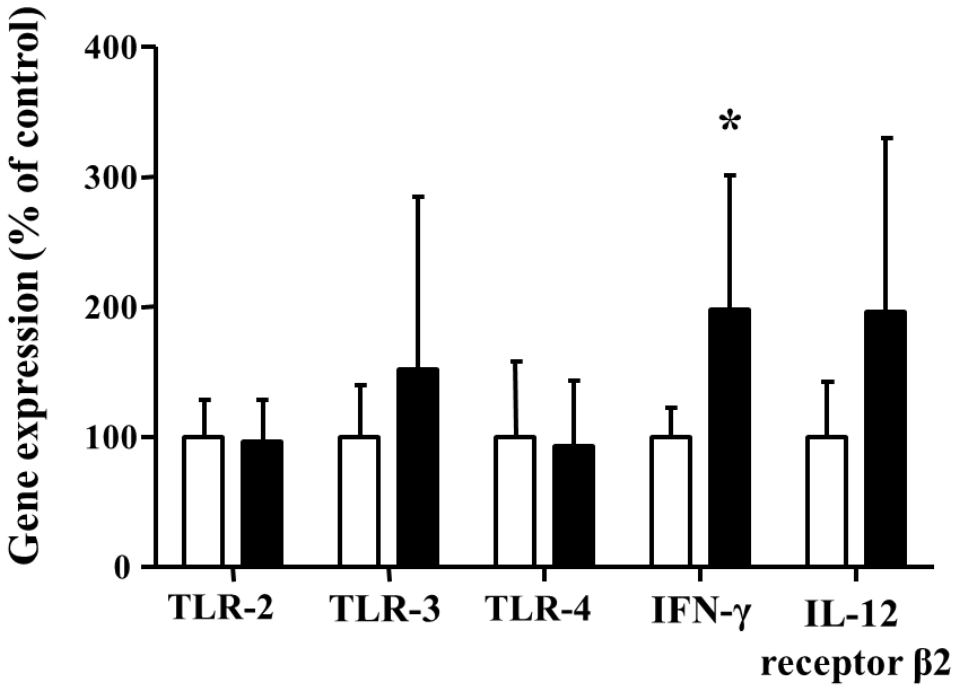

Figure 5-5 Effects of 06CC2 strain on gene expressions in Peyer's patches of IFV infected mice.

Effects of the administration of 06CC2 strain on gene expressions of mouse TLR 2, 3 and 4, IFN-γ, and IL-12 receptor β2 in Peyer's patches of IFV-infected mice (3 to 6 mice in a group) on day 2 after infection. The amounts of mRNA expressed from each gene were measured by real-time RT-PCR and normalized to the amounts of mouse β-actin mRNA. The levels of gene expression were expressed as the percentages of the control group. The data represent mean ± standard deviation. Open columns show the control group. Closed columns show the 06CC2-administered group.

5.4 Discussion

In this study, it was demonstrated that augmentation of NK cell activity associated with increases of pulmonary IFN-α, IL-12, and IFN-γ productions due to the oral administration of *L. plantarum* 06CC2 strain correlated with the alleviation of influenza infection in mice. The increases of pulmonary IFN-γ and IL-12 productions also correlated with the augmentation of IFN-γ and IL-12 receptor mRNA expression in Peyer's patches. These results suggested that the 06CC2 strain elicited immunomodulatory activity through intestinal immunity in IFV-infected mice. Previously, it was demonstrated that oral administration of *L. casei* Shirota ameliorated IFV infection in mice in association with increases of pulmonary NK cell activity and IL-12 production by mediastinal lymph node cells (Hori *et al*. 2002; Yasui *et al*. 2004). In addition, Maeda *et al*. (2009) reported that oral administration of the *L. plantarum* L-137 strain increased protection against IFV infection in mice by increasing IFN-β production in the serum. These findings using *Lactobacillus* species strain are consistent with my study using *L. plantarum* 06CC2, which modulated the host defense immunity including production of cytokines in IFV-infected mice. However, in this study, although the *L. delbrueckii* ssp. *lactis* 06TC3 strain, which is a different species from *L. plantarum* 06CC2, was effective in preventing the body weight loss of infected mice, *L. plantarum* 05AM23, 06TCa8, 06TCa40, and 06CC9, which are the same species as 06CC2, were not (Fig. 5-1A). Thus, the immunomodulatory activity of LAB, even in the same species and subspecies, varied, although the *L. plantarum* 06CC2 strain was characterized as a potent immunomodulator that is capable of controlling influenza infection.

I used a dose of 20 mg/mouse LAB as a maximum dose for mice to evaluate the possible immunomodulatory activity. The dose was deduced from use as a supplemental food by humans and corresponded to about 12-fold the dose for humans based on body surface area

(Freireich *et al*. 1966; Shimizu *et al* 2008). Oral administration of the 06CC2 strain did not produce significant body weight loss in mock-infected mice compared with the control and was significantly effective in protecting the body weight loss of infected mice and in prolonging survival times (Figs, 5-1B and 5-1C). Among 10 LAB strains examined, the 06CC2 strain was prophylactically effective in alleviating influenza symptoms without toxicity. Oral administration of the 06CC2 strain was also effective in reducing virus titers in lungs on days 2, 4 and 6 (Fig. 5-2). Because the boiled 06CC2 strain had no anti-influenza virus activity in a plaque-reduction assay using MDCK cells (data not shown), the reduction of virus titers on days 2, 4 and 6 after infection might result from elimination of the virus immunologically activated by the oral 06CC2 administration. This may be a part of the biological activity of the 06CC2 strain as an immunomodulator in hosts.

Oral administration of the 06CC2 strain augmented IFN-α production on day 2 after infection. Maeda *et al*. (2009) reported that oral administration of heat-killed *L. plantarum* L-137 enhanced protection against IFV infection in correlation with an increase in IFN-β production in the serum of infected mice at an early stage after infection. However, less IFN-β was detected in the BALF and serum of IFV-infected mice in this study (data not shown). In this study, IFN-α production was dominant in IFV-infected mice. Macrophages, monocytes, and neutrophils are the major producers of IFN-α, and they were probably responsible to IFN-α production in this murine IFV infection model.

IL-12 and IFN-γ productions as well as IFN-α production were augmented by 06CC2 administration in BALF from infected mice on day 2 after infection. Macrophages and monocytes are the major producers of IL-12 and IFN-γ as well as IFN-α (Fritz *et al*. 1999). IL-12 and IFN-α are strong inducers of IFN-γ and also induce cytotoxic activity of NK cells, which can induce IFN-γ (Robertson *et al*. 1992; Kos & Engleman 1996). In this study, the increase in IL-12 and IFN-γ productions due to 06CC2 administration on day 2 suggested that

NK cells were activated in the lungs of infected mice. NK cell activity in mice elicited by IFV infection was increased not only in the lungs but also in the spleens (Stein-Streilein *et al*. 1983; Ritz *et al*. 2006; Culley 2009). Actually, I demonstrated that NK activity in the spleen cells of infected mice on day 2 after 06CC2 administration was significantly higher than that in the control mice (Fig. 5-4). It is likely that oral administration of the 06CC2 strain activated NK cells through the augmentation of IL-12 and IFN-γ productions responding to the IFN-α that was produced by IFV infection in mice.

Oral administration of the 06CC2 strain significantly reduced the rise of TNF-α production in the BALF of infected mice on day 2 after infection. TNF-α is a proinflammatory cytokine, and the significant reduction was suggested to result in the suppression of inflammation early after IFV infection (Kurokawa *et al*. 2010). In the BALF of infected mice on day 2 after 06CC2 administration, the numbers of infiltrated cells, especially macrophages and neutrophils, were significantly reduced as compared with those in the control mice (Table 2). It is reported that macrophages and neutrophils markedly infiltrate the lungs of IFV-infected mice in the early phase of infection and that the excessive infiltration of cells causes the overreaction of host immune responses leading to lung consolidation and pneumonia (Oda *et al*. 1989; Kurokawa *et al*. 2010). Those findings also supported the hypothesis that the reduction of virus titer in lungs and the early suppression of inflammation after 06CC2 administration alleviated the influenza symptoms of IFV infection. Therefore, the activation of NK cells through the modulation of cytokine productions after 06CC2 administration probably contributed to the reduction of virus titer and the suppression of inflammation due to IFV infection in the lung of infected mice, resulting in the protection of body weight loss of infected mice in the early phase of infection.

The rise of IL-6 level in the BALF of IFV-infected mice was significantly suppressed by 06CC2 administration on day 6 after infection. This suggests that inflammation in lungs due

to IFV infection was weakened on day 6. The virus titers in the lungs of infected mice after 06CC2 administration were obviously lower than those in the control mice on day 6, suggesting that virus-elimination was promoted by 06CC2 administration. It is possible that the NK cells activated by 06CC2 administration eliminated IFV-infected cells in the early phase of infection, resulting in the reduction of virus yields in the later phase.

IL-12 and IFN-γ productions in the BALF from IFV-infected mice on day 2 after infection were augmented by 06CC2 administration, but the numbers of infiltrated macrophages and neutrophils were reduced. These results suggested that infiltrated cells producing IL-12 and IFN-γ, mainly macrophages including dendritic cells, were qualitatively activated by 06CC2 administration. Peyer's patches are an intestinal immune organ and it is possible that the 06CC2 strain first gives an influence on host immune system. It was demonstrated that the gene expressions of IFN-γ and IL-12 receptor in Peyer's patches were selectively augmented by 06CC2 administration (Fig. 5-5). However, 06CC2 administration did not affect the gene expressions of TLRs 2, 3, and 4 in Peyer's patches of infected mice on day 2, suggesting that the gene expressions of IFN-γ and IL-12 receptor by 06CC2 administration might not be associated with innate immunity through the gene expressions of TLRs 2, 3, and 4 in Peyer's patches. Tsai *et al*. (2010) reported that the administration of *L. paracasei paracasei* NTU 101 enhanced the gene expressions of IL-12 and IFN-γ and $CD4^+$ T cell-dendritic cell interaction in Peyer's patches but not in spleen cells. My findings were consistent with their results and suggested that macrophages and dendritic cells activated by the 06CC2 strain in Peyer's patches were responsible to the augmentation of IL-12 and IFN-γ production in the BALF from IFV-infected mice followed by the enhancement of NK cell activity.

Generally, LAB is taken orally as a foodstuff. Previous reports described the immunological reactions between microorganisms including probiotics and the host immune system in the intestine (Corthésy *et al*. 2007; Winkler *et al*. 2007; O'Flaherty &

Klaenhammer. 2010). Understanding how intestinal immunity is involved in the alleviation of influenza symptoms is important. In this study, it was demonstrated that the 06CC2 strain exhibited immunomodulatory activity in IFV-infected mice, resulting in the alleviation of IFV infection and investigated the mechanism of action by administration 06CC2 strain. The 06CC2 strain as a supplemental food might be useful as a possible probiotic to alleviate IFV infection prophylactically.

5.5 Conclusion

In this study, the immunomodulatory activity of 10 probiotics strains from Mongolian dairy products was assessed on IFV infection in relation to their efficacies in IFV infected mice. Oral administration of boiled 06CC2 strain, one of the 10 LABs, was significantly effective on not only the symptoms of infected mice but also reducing virus yields in the lungs after infection without toxicity. Also, I found that 06CC2 was suggested to alleviate influenza symptoms in mice in correlation with the augmentation of NK cell activity associated with the enhancement of IFN-α and Th1 cytokine productions through intestinal immunity and the reduction of TNF-α in the early stage of infection. Therefore, the 06CC2 strain exhibited immunomodulatory activity in IFV-infected mice, resulting in the alleviation of IFV infection. The development of functional dairy products using the 06CC2 strain such as yoghurt that could prevent IFV infection or to attenuate IFV infectious syndrome was anticipated.