RESEARCH ARTICLE

Oral Administration of *Lactococcus lactis* Subsp. *lactis* JCM5805 Enhances Lung Immune Response Resulting in Protection from Murine Parainfluenza Virus Infection

Kenta Jounai¹,²*, Tetsu Sugimura², Konomi Ohshio², Daisuke Fujiwara²

¹ Technical Development Center, Koiwai Dairy Products Co Ltd. Sayama, Japan, ² Central Laboratories for Key Technologies, Kirin Co. Ltd., Yokohama, Japan

* Kenta_Jounai@kirin.co.jp

Abstract

When activated by viral infection, plasmacytoid dendritic cells (pDCs) play a primary role in the immune response through secretion of IFN-α. *Lactococcus lactis* subsp. *lactis* JCM5805 (JCM5805) is a strain of lactic acid bacteria (LAB) that activates murine and human pDCs to express type I and type III interferons (IFNs). JCM5805 has also been shown to activate pDCs via a Toll-like receptor 9 (TLR9) dependent pathway. In this study, we investigated the anti-viral effects of oral administration of JCM5805 using a mouse model of murine parainfluenza virus (mPIV1) infection. JCM5805-fed mice showed a drastic improvement in survival rate, prevention of weight loss, and reduction in lung histopathology scores compared to control mice. We further examined the mechanism of anti-viral effects elicited by JCM5805 administration using naive mice. Microscopic observations showed that JCM5805 was incorporated into CD11c⁺ immune cells in Peyer’s patches (PP) and PP pDCs were significantly activated and the expression levels of IFNs were significantly increased. Interestingly, nevertheless resident pDCs at lung were not activated and expressions levels of IFNs at whole lung tissue were not influenced, the expressions of anti-viral factors induced by IFNs, such as *Isg15*, *Oasl2*, and *Viperin*, at lung were up-regulated in JCM5805-fed mice compared to control mice. Therefore expressed IFNs from intestine might be delivered to lung and IFN stimulated genes might be induced. Furthermore, elevated expressions of type I IFNs from lung lymphocytes were observed in response to mPIV1 *ex vivo* stimulation in JCM5805-fed mice compared to control. This might be due to increased ratio of pDCs located in lung were significantly increased in JCM5805 group. Taken together, a specific LAB strain might be able to affect anti-viral immunological profile in lung via activation of intestinal pDC leading to enhanced anti-viral phenotype *in vivo*. 
Introduction

Probiotics are live microorganisms in intestinal flora or starter cultures for dairy products that have been reported to have beneficial effects on human health. Lactic acid bacteria (LAB) had been reported to produce potent and diverse immunomodulatory effects. Among these, the protective effect of LAB against viral infection is of particular interest. For example, oral administration of *Bifidobacterium breve* YIT4064 has been shown to be effective against rotavirus-induced diarrhea and influenza virus infection [1,2]. *Lactobacillus pentosus* S-PT84, *L. plantarum* 06CC2, *L. acidophilus* L-92, *Enterococcus faecalis* FK-23 and *L. casei* shirota have also been reported to protect mice from influenza virus infection [3–8]. However, little is known about the mechanism of the effect of LAB on viral infection.

Plasmacytoid dendritic cells (pDCs) act in the innate immune system as the first line of defense against viral infection and, triggered by viral nucleic acids, secrete a large amount of interferon-α (IFN-α) [9,10]. Recently, pDCs have been shown to be important not only as a producer of IFNs but also as a regulatory cell that control various immune subsets, such as CD4+ / CD8+ T cells and B cells [11,12]. Takagi et al. have shown that pDCs suppress induction of the CD4+ T cell response and participate in initiation of CD8+ T cells against virus, using Siglec-H-deficient mice and pDCs-ablated mice [13]. pDCs also play a prominent role in mucosal immunoglobulin A (IgA) productions by expression of APRIL and BAFF [14]. Therefore, stimulation of host pDCs is considered to have a protective effect against viral infection.

Although some pathogenic bacteria (e.g., *Staphylococcus aureus*) have been shown to stimulate pDCs [15], beneficial bacteria (e.g., LAB) have been shown to be ineffective in stimulating pDCs [16]. However, we screened non-pathogenic LAB strains and found that LAB strain JCM5805 stimulated murine pDCs to produce Type I and III IFNs in association with myeloid dendritic cells [17]. JCM5805 was also shown to activate human pDCs isolated from peripheral blood mononuclear cells (PBMCs) *in vitro* and administration of JCM5805 significantly affected pDC activity in humans [18].

Murine parainfluenza virus type 1 (mPIV1) is a member of the family *Paramyxoviridae*: enveloped, negative-strand, single-stranded RNA (ssRNA) viruses. mPIV1 ssRNA is recognized by Toll-like receptor (TLR) 7 and TLR8 [19], which are highly expressed in pDCs, and the RIG-I helicase in the cytosol recognizes viral nucleic acids in conventional dendritic cells (cDCs) and fibroblasts [20–22]. mPIV1 has been reported to induce acute lung inflammation in mice, that are widely used as a respiratory viral infection model [23]. Because of its robust proliferation in embryonated eggs and cell cultures, mPIV1 has been extensively studied [24]. mPIV1 is highly infectious and induces pathological lesions in the lung, leading to lethality in mice [25].

Using the mPIV1-infected mouse model, we studied the preventive effects of oral administration of JCM5805 against viral infection. We also investigated the mechanism of activation of anti-viral immunity induced by oral administration of JCM5805 by examining immunity in intestine and lung tissues using naive mice. As a result, we found that oral administration of JCM5805 protected mice against mPIV1 infection by activating an immune response in lung tissue by stimulating pDCs localized in the intestine.

Materials and Methods

Mice

For studies of the anti-viral effects of JCM5805 administration in mPIV1-infected mice, 6- to 10-weeks-old female wild-type DBA/2Jcl mice were purchased from CLEA Japan. DBA/2J mice are used for experiment of mPIV1 infection, because its susceptibility against mPIV1.
Mice were divided equal average weight into two groups. Control group mice (n = 18) were fed AIN93G (Oriental Yeast, Tokyo, Japan) and JCM5805 group mice (n = 19) were fed AIN93G containing of 1 mg heat-killed JCM5805 / day / mouse and water ad libitum. The mice were housed three per cage in specific pathogen-free conditions under a 12hr light / dark cycle. The temperature in the room was kept at 22 ± 2°C and 60 ± 15% humidity. The treatment was started 14 days before mPIV1 infection and continued for 15 days after infection. Animal procedures and experiments were approved by the Laboratory Animal Care Committee of the Central Institute for Experimental Animals. The approval ID of these experiments was CA1101.

For studies of the effect of JCM5805 administration on immunity in naive mice, 8- to 10-weeks old female C57BL/6J wild-type mice were purchased from Charles River Japan. Mice were divided equal average weight into two groups. Control group mice (n = 8) were fed AIN93G and JCM5805 group mice (n = 8) were fed AIN93G containing of 1 mg heat-killed JCM5805 / day / mouse and water ad libitum. The mice were housed one per cage in specific pathogen-free conditions under a 12hr light / dark cycle. The temperature in the room was kept at 25 ± 1°C and 60 ± 15% humidity. Animal procedures and experiments were approved by the Laboratory Animal Care Committee of Central Laboratories for Key Technologies, Kirin Co., Ltd. The approval ID of these experiments was YO11-00147. Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress. Animals were monitored for their conditions and the clinical scores were recorded every day after the mPIV1 infection. Animals surviving the infection were sacrificed anaesthetically at day 15 using diethyl ether.

mPIV1 infection

mPIV1 was prepared by the Central Institute for Experimental Animals. Mice were inoculated twice using a micropipette, with 4 hr between inoculations, by intranasal administration of a 25 μl drop containing 64 hemagglutination units (HAU) of mPIV1.

Body weight measurements

Net body weights were measured daily during the course of experiments of mPIV1 infection. Animals surviving the infection were weighed daily and those with severe weight loss of 25% or more were sacrificed anaesthetically.

General conditions and number of mice with emaciation after infection with mPIV1

General conditions and unusual condition of the animals were recorded daily by clinical observation. Number of mice with emaciation was evaluated every day after the mPIV1 infection for 15 days. The examination was performed by two independent observers with the same observation criteria to eliminate possible bias associated with individuals. The emaciation state was evaluated as following features: −: no emaciation; +: emaciation. Once the animals display humane endpoints, they are humanely killed.

Lung histopathology

At 3 days post-infection, six mice each from the control and JCM5805 groups were sacrificed anaesthetically and their lungs were taken from the mice the right lung was taken from six mice and fixed with 10% PFA. Fixed sections of paraffin-embedded lungs were stained with hematoxylin and eosin (H&E) (Sakura Finetek). Slides were randomized, read blindly, and examined
for tissue damage and inflammatory cellular infiltration. The observations were scored in four levels as follows: 0, no pathogenicity; 1, low pathogenicity; 2, medium pathogenicity; 3, high pathogenicity.

Microscopic observation of phagocytosis in Peyer’s patches
Fluorescent-labeled JCM5805 were prepared as follows: 30 mg JCM5805 / 1 ml FITC solution [0.1 mg FITC isomer 1 (Sigma) / ml 0.1 M NaHCO₃ buffer, pH 9.0] was incubated for 60 min at 25°C and then washed three times with PBS. To investigate phagocytosis by CD11c⁺ cells, C57BL/6J mice were orally administered FITC-labeled JCM5805 (30 mg/mouse), sacrificed anaesthetically 8 hr later, and their Peyer’s patches (PP) were excised. PP cells were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) and sliced into 6 μm sections. The sections were fixed in cold acetone (Wako) for 10 min at −20°C. Then the slides were washed with PBS containing 1% BSA and stained with affinity purified anti-CD11c (Thermo) for 2 hr at room temperature. The slides were washed again with PBS and stained with anti-rat IgG labeled with Alexa Fluor 546 (Invitrogen) for 30 min at room temperature. The slides were washed again with PBS and mounted with Fluoromount (Diagnostic Biosystems). Fluorescence microscopy was performed using an Olympus BX60.

Intestinal cell preparation and FACS analysis
PP were minced in Mg²⁺- and Ca²⁺-free Hank’s Balanced Salt Solution and digested with 1 mg collagenase (Sigma) / ml and 0.2 mg DNase 1 / ml for 20 min at 37°C. EDTA was added to 30 mM and the mixture was incubated for 10 min at room temperature. Tissue lysates were filtered through a 70 μm nylon cell strainer and layered onto 15% Histodenz (Sigma) in RPMI 1640 containing 10% FCS, and centrifuged at 450 x g for 20 min without braking. The low density fractions at interfaces were collected and washed. The cells were stained with a fluorescent dye conjugated to an antibody as follows: I-A / I-E-FITC (M5 / 114.15.2) (eBiosciences), CD3ε-PE (145-2C11) (eBiosciences), Siglec-H-APC (551.3D3) (Milteny Biotec), and CD11c-PE-Cy7 (N418) (eBiosciences). After staining, the cells were washed twice with FACS buffer (0.5% BSA in PBS buffer) and suspended in 4% paraformaldehyde (Wako) for FACS analysis. Data were collected by a FACS Canto II (BD Biosciences) and analyzed by FCS Express software (De Novo Software). CD3⁻CD11c⁺ Siglec-H⁺ cells were defined as pDCs, and the expression levels of cell surface markers on these pDCs were measured.

Gene expression analysis
Total RNA was extracted using an RNeasy Kit (Qiagen), and cDNA was prepared using an iScript cDNA synthesis kit (BioRad), according to the manufacturer’s protocol. Quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa) and a LightCycler 480 (Roche). The methods and primers for qRT-PCR of Gapdh, Ifna, Ifnβ, Isg15, Oasl2, Viperin and Cxcl12 have been previously described [26,27]. mRNA expression of GAPDH was used as internal control for normalization of gene expression analysis. The nucleotide sequences of primers were follows: Gapdh forward (F) (AACGACCCCTTCATTGAC) and Gapdh reverse (R) (TCCACGACATACTCAGCAG), Ifna F (AGCAGGTGGGGGTGGCAGA) and Ifna R (ACCACCTCCAGGCAGGGA), IfnβF (TCAGAATGAGTGTTGTTGC) and Ifnβ R (GACCTTTCAATTCAGTACGC), Isg15 F (GAGCTAGCGCTGACAAAT) and Isg15 R (TTCTGGGCAATCTGCTTCTG), Oasl2 F (CGATGCGGAGAGGAGGAA) and Oasl2 R (TGCCCTGCTTCTGGAACGT), and Viperin F (CTTCCAGCTGGAGGGAAGA) and Viperin R (GAGCTCTGACACACTTCTG) and Cxcl12 F (GGGGTCATCGAGCATTC) and Cxcl12 R (CCGGAATGCGAGCACCTGTC).
Expression of IFNs against inactivated mPIV1 ex vivo

Single-cell suspensions of lung samples were prepared as previously described [28]. Lung lymphocytes were cultured at a density of $1 \times 10^6$ cells/ml RPMI medium in 48 well plates for 24 hr at 37°C, with or without 0.5 AU inactivated mPIV1/ml. The concentrations of IFN-α and IFN-β in culture supernatants were analyzed by ELISA (PBL Biomedical Laboratories).

Statistics

Statistical differences between two groups were determined using an unpaired, two-tailed Student’s t test with significance set at $P<0.05$. For survival studies, a Log-Rank (Mantel-Cox) test was used. Clinical scores were evaluated using a chi-square test. For lung histopathology, the Mann-Whitney U test was used.

Results

Effect of JCM5805 on mPIV1 infection

mPIV1 infection of the mouse lung causes pathological lesions leading to lethality [25]. The mouse mPIV1 infection model was used to study the effect of JCM5805 on viral infection by feeding a diet containing JCM5805 starting 14 days before mPIV1 infection (Fig. 1A). Six mice from the control and JCM5805 groups were sacrificed anesthetically 3 days after infection and the lungs were isolated to examine histopathology.

Fig. 1B shows the survival rates of mice in the control and JCM5805 groups up to 15 days after mPIV1 infection. Animals surviving the infection were weighed daily and those animals with severe weight loss of 25% or more were sacrificed anesthetically and regarded as dead animals. The survival rate of control group mice was 50.0% at day 8, 8.3% at day 9, and 0% at day 10, whereas for JCM5805 group mice it was 92.3% at day 8, 76.9% at day 9, and 69.2% at day 11. Therefore, there was a drastic increase in the survival rate ($P<0.001$) of JCM5805 group mice compared to control group mice.

As shown in Fig. 1C, the body weight of mPIV1-infected control group mice showed a continuous decrease throughout the experiment. In contrast, the body weight of mPIV1-infected JCM5805 group mice decreased slightly for a few days after infection, then leveled off for a few days and finally increased slightly through the end of the experiment.

Table 1 shows the number of mice with emaciation after infection. JCM5805 group mice showed significantly lower number of mice emaciation after day 6 compared to control group.

Fig. 2A shows microscopic fields of lung tissue from mPIV1-infected mice in each group. Control group mice had extensive lung damage; i.e., highly basophilic epithelium lining the bronchioles, focal degenerating cells undergoing apoptosis or necrosis, and extensive cellular infiltrates of neutrophils, monocytes and lymphocytes. However, JCM5805 group mice showed decreased prevalence of epithelial cells with morphologic features of degeneration and necrosis (Fig. 2B). These data strongly indicated that oral administration of JCM5805 before mPIV1 infection was highly effective in preventing and/or reducing viral pathogenicity.

Fluorescence microscopy of JCM5805 incorporation into PP

We then used naive mice to investigate the mechanism by which JCM5805 may affect viral pathogenicity. FITC-labeled JCM5805 was orally administrated to mice and frozen sections of PP were stained by CD11c antibody and visualized by fluorescence microscopy. As shown in Fig. 3A and B (magnified image of white boxed area of Fig. 3A), FITC-labeled JCM5805 was incorporated into CD11c+ immune cells in the subepithelial dome (SED) area and was also found inside the small intestinal lamina propria (Fig. 3C).
Fig 1. Effects of JCM5805 on mPIV1 infection. A. Experimental procedure of mPIV1 infection. Mice in the control and JCM5805 groups were fed diet with or without 1 mg / mouse / day of JCM5808 during the study period (day -14 to 15). Mice were intranasally infected with mPIV1 on day 0. On 3 days post-mPIV1 infection, six mice were sacrificed from each group for lung histopathology. Thereafter survival rate, body weight and clinical scores were investigated with remained control mice n = 12, and JCM5805 mice n = 13. B. Survival rate of mice infected with mPIV1. The control (circle) and JCM5805 (square)
Activation of pDCs in intestine by JCM5805 administration

To examine the activation of pDCs in PP after JCM5805 incorporation, status of pDCs were analyzed in PP by FACS two weeks after JCM5808 administration. Expression of MHC class II on pDCs in JCM5808 group mice was significantly greater than in control group mice (Fig. 4A). However, ratio of pDCs in JCM5805 group mice was not significantly affected compared to control group. We next examined expression level of type I IFNs from pDC located in PP. Both Ifnα and Ifnβ mRNA levels in PP pDCs prepared from JCM5805 group were significantly greater than control group (Fig. 4B). These data suggested that JCM5805 was taken up from the intestinal tract through PP and activated resident pDCs to increase type I IFNs at draining mucosal sites.

Effects of JCM5805 on anti-viral immunity at lung

Little is known about the effect of orally administrated LAB on systemic and local immunity or about how host-microbe crosstalk affects the immune response in peripheral tissues, such as lung. To determine whether oral administration of JCM5805 affects resident pDCs at lung,

Table 1. Numbers of mice with emaciation after infection with mPIV1.

| Days after infection | Group     | Number of mice with emaciation | Chi-square test |
|---------------------|-----------|--------------------------------|-----------------|
|                     |           | −                              | +               |                |
| 3                   | Control   | 11                             | 1               | P = 0.288      |
|                     | JCM5808   | 13                             | 0               |                |
| 4                   | Control   | 11                             | 1               | P = 0.288      |
|                     | JCM5805   | 13                             | 0               |                |
| 5                   | Control   | 9                              | 3               | P = 0.548      |
|                     | JCM5805   | 11                             | 2               |                |
| 6                   | Control   | 1                              | 11              | **P < 0.01     |
|                     | JCM5805   | 11                             | 2               | **P < 0.01     |
| 7                   | Control   | 0                              | 12              | **P < 0.01     |
|                     | JCM5805   | 10                             | 3               | **P < 0.01     |
| 8                   | Control   | 0                              | 6               | **P < 0.01     |
|                     | JCM5805   | 9                              | 3               | **P < 0.01     |
| 9                   | Control   | 0                              | 1               | *P < 0.05      |
|                     | JCM5805   | 9                              | 1               | *P < 0.05      |
| 10                  | Control   | 0                              | 1               | *P < 0.05      |
|                     | JCM5805   | 9                              | 1               | *P < 0.05      |

The emaciation state of mice in the control and JCM5805 groups were evaluated after mPIV1 infection. The emaciation state was evaluated as follows: −, no emaciation; +, emaciation. *P < 0.05
**P < 0.01 (Chi-square test). The data shown is representative of two independent experiments.

doi:10.1371/journal.pone.0119055.t001
pDCs status and IFNs-related gene expressions were evaluated using JCM5805-fed mice. FACS analysis revealed that the expression level of activation marker, MHC class II, was not changed. However, ratio of pDCs at lung tissue derived from JCM5805 group was increased compared to control (Fig. 5A). Next, expression level of IFNs were not changed between two groups when it was compared using RNA prepared from whole tissue due to limited number of pDCs at lung (Fig. 5B). However, interestingly, expressions level of IFN-related genes, *Isg15*, *Oasl2*

Fig 2. Lung histopathology of mPIV1-infected mice. A. Representative hematoxylin and eosin (H & E)-stained sections of lung tissues from control and JCM5805 group mice (6 mice per group). Lung tissues were prepared from mice 3 days after infection. Scale bars, 300 μm. B. Histological scoring of lung tissues from mPIV1-infected mice belong to control (open columns) and JCM5805 (dot columns) group. Sections were scored at four levels as follows: 0, no symptoms; 1, low pathogenicity; 2, medium pathogenicity; 3, high pathogenicity. The mean ± SD of the tissues in each group is shown. *P*<0.05, **P**<0.01 (Mann-Whitney U test). The data shown is representative of two independent experiments.
Fig 3. Fluorescence microscopy observation of intestine derived from JCM5805-administered mice. PPs were removed 8 hr after oral administration of FITC-labeled JCM5805 (green). (A, B) Observations at PP sections where CD11c was labeled with anti-CD11c (red). Magnifications of microscopic images are (A) × 200 and (B) × 600. (C) Observation at intestinal villi. The original magnification is × 100. These data are representative from two experiments that yielded similar results.

doi:10.1371/journal.pone.0119055.g003
and Viperin were greater in lung whole tissues of JCM5805 group compared to control group (Fig. 5C). These data may imply that expressed IFNs by PP pDCs after oral administration of JCM5808 might be dispersed throughout of the whole body including lung and as a consequence expression of IFN-related genes in lung might be induced. Furthermore we sought

Fig 4. Activation of pDCs in intestine by JCM5805 administration. Healthy C57BL/6J mice were divided into control and JCM5805 groups (n = 4 in each group), and mice in the JCM5805 group were orally administered JCM5808 daily for 2 weeks. A. Low density cells prepared from PP of each group were analyzed by FACS. Expression level of cell surface activation marker was evaluated for MHC class II as median fluorescence intensities (M.F.I.) in left panel. Ratio of pDCs to total population was shown in right panel. pDCs was defined as “CD3− Siglec-H+ CD11c+” in total population. Short line represents the mean values. *P<0.05 (Student’s t test). B. Total mRNA was extracted from PP pDCs from mice in the control (open columns) and JCM5805 groups (dot columns) (n = 8 in each group). Ifnα and Ifnβ gene expressions were measured by qRT-PCR and normalized to Gapdh gene expression. Data are shown as mean ± SD. *P<0.05 (Student’s t test). These data are representative of three independent experiments. Each data are mean ± SD.

doi:10.1371/journal.pone.0119055.g004
Fig 5. Effects of JCM5805 administration on lung immunity. Healthy C57BL/6J mice were divided into control and JCM5805 groups (n = 8 in each group), and then mice in the JCM5805 group were orally administered JCM5805 daily for 2 weeks. A. Low density cells prepared from lung of each group were analyzed by FACS. Expression level of cell surface activation marker was evaluated for MHC class II as M.F.I. in left panel. Ratio of pDCs to total population was shown in right panel. pDCs was defined as "CD3− Siglec-H+ CD11c+ in total population". Short line represents the mean values. **P<.01
whether lung immune response against mPIV1 might be affected by JCM5805 administration, lung lymphocytes derived from control and JCM5805 group were cultured with inactivated mPIV1 _ex vivo_ and the expressions of IFN-α and IFN-β were measured. As a result, both IFN-α and IFN-β expressed by lung lymphocytes from JCM5805 group were significantly elevated compared to control group (Fig. 5D). This might be reflected by increased pDCs ratio at lung in JCM5805 group. Taken together, it was strongly suggested that anti-viral immunity at lung was indeed affected by JCM5805 administration via pDCs activation at intestine.

### Discussion

Since direct activation of pDCs and induction of type I and III IFNs by LAB have been shown to be minimal [17], a mouse model of mPIV1 infection was used in this study to evaluate the antiviral effect of LAB JCM5805 administration. As a result, it was shown that a significant improvement, such as survival rate, body weight and histopathology scores of lung tissues, were observed in JCM5805 group compared to control.

A number of studies have reported that LAB have some beneficial anti-viral effects against influenza virus in mice [3–8], by oral administration of _L. plantarum_ 06CC2 (10 mg/day/mouse) or _L. acidophilus_ L-92 (10 mg/day/mouse), or intranasal administration of _L. pentosus_ S-PT84 (20 or 200 μg/day/mouse) or _L. casei_ shirota (20 or 200 μg/day/mouse). These anti-viral effects of LABs are consistent with activation of classical innate immunity, such as NK cells and macrophages.

pDCs have been shown to contribute to host anti-viral defense through multiple mechanisms. Not only pDCs secrete type I IFN, but also they secrete IL-12 to induce Th1 polarization of CD4+ T cells [29]. pDCs are also able to destroy virus-infected cells in a FasL- and TRAIL-dependent manner [30,31]. Recently, Takagi _et al._ have shown that pDCs are responsible for generation of virus-specific CD8+ T cells by using inducible ablation mice model of pDCs _in vivo_ [13]. In addition, Swiecki _et al._ showed that pDCs play a crucial role in generating virus-specific CD8+ T cells and also in expansion of virus-specific NK cells in response to murine cytomegalovirus and vesicular stomatitis virus infection [32]. These reports strongly suggested that pDCs is important subset, not only in terms of early host response against virus infection as a member of innate immunity, which is a major source of type I IFN, but also in late host response by linking with virus-specific cytotoxic T cell expansion _in vivo_. Therefore, JCM5805 may be a unique and effective anti-viral therapy by stimulating both the adaptive and innate immune systems.

We also demonstrated that JCM5805 was taken up by CD11c+ cells in PP. The surface of PP is covered with specialized follicle-associated epithelia (FAE), where various antigens are captured by M cells [33]. Some _Lactobacillus_ strains have been reported to be incorporated into CD11c+ and CD11b+ cells in PP [34,35], and JCM5805 was shown to be incorporated into CD11c+ cells in PP in this study. Recently, other routes, such as intestinal villous M cells [36] and CX3CR1+ DCs [37,38], have been reported to be novel antigen entry sites in the mucosal epithelium. In agreement with those reports, we also found that JCM5805 was taken up by the intestinal villus. Therefore, JCM5805 might stimulate intestinal pDCs using both PP and the...
intestinal villus as entry sites. DCs in PP can be divided into four populations: CD11c^{high} CD11b^{−} CD8α^{+} DCs (CD11b^{−} DCs), CD11c^{high} CD11b^{−} CD8α^{+} DCs (CD8α^{+} DCs), CD11c^{high} CD11b^{−} CD8α^{+} DCs (DNDCs) and pDCs [39]. In this study, oral administration of JCM5805 was shown to activate pDCs in PP and induce expression of type I IFNs. Although the role of PP pDCs in the immune response is not fully understood, it has been reported that pDCs in GALT enhance the suppressive efficacy of CD4^{+} CD25^{+} Treg generation and might contribute to immune tolerance and gut homeostasis [40]. Since we have shown that enhanced Treg generation is occurred by JCM5805-treated pDCs in vitro [17], oral administration of JCM5805 might also contribute to the maintenance of gut homeostasis in vivo.

mPIV1 has been reported to induce acute lung inflammation in mice, which has been used as a respiratory viral infection model [23]. One of the biological effects of IFNs is induction of expression of IFN-stimulated genes (ISGs) that are involved in inhibition of viral replication and release. Expressions of three important ISGs (Isg15, Oasl2 and Viperin) were examined in this study. Isg15 inhibits replication and release of influenza virus and type I herpes simplex virus from infected cells [41], Oasl2 is a 2′-5′-oligoadenylate synthetase that activates RNase L and degrades viral RNAs [41–43] and Viperin is a multifunctional antiviral factor that inhibits the growth of DNA and RNA viruses; e.g., hepatitis C virus, cytomegalovirus, influenza virus and flaviviruses [44,45]. In this study, oral administration of JCM5805 increased expression of these three ISGs in lung tissue, which is distant from JCM5808 entry site. This local increase of expression of anti-viral factors at lung may have contributed to the drastic increase in survival rate of JCM5808 group in mPIV1 infection experiment. Recently, Ichinohe et al. showed that host commensal microbiota composition critically regulates the generation of virus-specific CD4^{+} and CD8^{+} T cells and the antibody response in lung tissue following respiratory influenza virus infection [46]. Abt et al. reported that intestinal microbiota induces IFN-β production from peritoneal and alveolar macrophages via phosphorylation of STAT1 and, as a result, expression of ISGs in lung tissue was elevated in mice infected by influenza virus [47]. These reports and our observation might imply that there is a close association between bronchus-associated lymphoid tissue (BALT) and GALT. We observed statistically significant increased expression of Ifna and Ifnb in PP pDCs in JCM5805 group compared to control, however there were not statistically significant change in lung tissue (Ifna, P = 0.17, Ifnb, P = 0.22.) Therefore, IFNs produced in intestine might affect expression of ISGs in lung tissue via the blood circulation system. Interestingly, the response of lung lymphocytes prepared from JCM5805 group against mPIV1 was up-regulated compared to control. We observed increased ratio in resident pDCs at lung by JCM5805 administration, therefore the increased response by lung lymphocytes derived JCM5805 group may due to increased number of pDCs located at lung. It is intriguing that chemoaattraction in lung might be affected by JCM5805 administration, therefore expression of CXCL12, which is reported to involved in migration of pDCs [48], were examined at lung. However no change was observed in CXCL12 (data not shown), other molecules involved in pDC chemoaattraction might be changed by JCM5805 administration.

In conclusion, oral administration of JCM5805 was shown to elicit a significant anti-viral response against respiratory viral infection via enhancing lung immune response through activation of pDCs in the intestine in this study. Since we are regularly exposed to potential infectious threats, safe and effective immunomodulatory agents are widely required. LABs are generally accepted as safe food agents, specific LABs that affect pDCs might be useful and novel immune adjuvant that is able to increase systemic immune response by activating intestinal pDCs.
Acknowledgments
We thank Dr. Masachika Okamura and Dr. Yasuyuki Tomita for their valuable discussion.

Author Contributions
Conceived and designed the experiments: KJ DF. Performed the experiments: KJ TS KO. Analyzed the data: KJ KO. Wrote the paper: KJ DF.

References
1. Yasui H, Kiyoshima J, Ushijima H (1995) Passive protection against rotavirus-induced diarrhea of mouse pups born to and nursed by dams fed Bifidobacterium breve YIT4064. J Infect Dis 172: 403–409. PMID: 7622883
2. Yasui H, Kiyoshima J, Hori T, Shida K (1999) Protection against influenza virus infection of mice fed Bifidobacterium breve YIT4064. Clin Diagn Lab Immunol 6:186–192. PMID: 10066652
3. Izumo T, Maekawa T, Ida M, Noguchi A, Kitagawa Y, et al. (2010) Effect of intranasal administration of Lactobacillus pentosus S-PT84 on influenza virus infection in mice. Int Immunopharmacol 10: 1101–1106. doi: 10.1016/j.intimp.2010.06.012 PMID: 20601181
4. Takeda S, Takeshita M, Kikuchi Y, Dashnyam B, Kawahara S, et al. (2011) 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. Int Immunopharmacol 11: 1976–1983. doi: 10.1016/j.intimp.2011.08.007 PMID: 21871585
5. Goto H, Sagitani A, Ashida N, Kato S, Hirota T, et al. (2013) Anti-influenza virus effects of both live and non-live Lactobacillus acidophilus L-92 accompanied by the activation of innate immunity. Br J Nutr 110: 1810–1818. doi: 10.1017/S0007114513001104 PMID: 23594927
6. Kondoh M, Fukada K, Fujikura D, Shimada T, Suzuki Y, et al. (2012) Effect of water-soluble fraction from lysozyme-treated Enterococcus faecalis FK-23 on mortality caused by influenza A virus in mice. Viral Immunol 25: 86–90. doi: 10.1089/vim.2011.0056 PMID: 22252469
7. Hori T, Kiyoshima J, Shida K, Yasui H (2002) Augmentation of cellular immunity and reduction of influenza virus titer in aged mice fed Lactobacillus casei strain Shirota. Clin Diagn Lab Immunol 9: 105–108. PMID: 11777838
8. Hori T, Kiyoshima J, Shida K, Yasui H (2001) Effect of intranasal administration of Lactobacillus casei Shirota on influenza virus infection of upper respiratory tract in mice. Clin Diagn Lab Immunol 8: 593–597. PMID: 11329464
9. Theofilopoulos AN, Baccala R, Beutler B, Kono DH (2005) Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol. 23: 307–336. PMID: 15771573
10. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, et al. (2004) Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 101: 5598–5603. PMID: 15034168
11. Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, et al. (2003) Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat Immunol 4: 1009–1015. PMID: 14502286
12. Marrack P, Kappler J, Mitchell T (1999) Type I interferons keep activated T cells alive. J Exp Med 189: 521–530. PMID: 9927514
13. Takagi H, Fukaya T, Eizumi K, Sato Y, Sato K, et al. (2011) Plasmacytoid dendritic cells are crucial for the initiation of inflammation and T cell immunity in vivo. Immunity 35: 958–971. doi: 10.1016/j.immuni.2011.10.014 PMID: 22177923
14. Tezuka H, Abe Y, Asano J, Sato T, Liu J, et al. (2011) Prominent role for plasmacytoid dendritic cells in mucosal T cell-independent IgA induction. Immunity 34: 247–257. doi: 10.1016/j.immuni.2011.02.002 PMID: 21335555
15. Parcina M, Wendt C, Goetz F, Zawatzky R, Zahringer U, et al. (2008) Staphyloccoccus aureus-induced plasmacytoid dendritic cell activation is based on an IgG-mediated memory response. J Immunol 181: 3823–3833. PMID: 18768836
16. Piccioli D, Sammicheli C, Tavarini S, Nuti S, Frigimelica E, et al. (2009) Human plasmacytoid dendritic cells are unresponsive to bacterial stimulation and require a novel type of cooperation with myeloid dendritic cells for maturation. Blood 113: 4232–4239. doi: 10.1182/blood-2008-10-186890 PMID: 19176317
17. Jounai K, Ikado K, Sugimura T, Ano Y, Braun J, et al. (2012) Spherical lactic acid bacteria activate plasmacytoid dendritic cells immunomodulatory function via TLR9-dependent crosstalk with myeloid dendritic cells. PLoS One 7: e32586. doi: 10.1371/journal.pone.0032586 PMID: 22505996
18. Sugimura T, Jounai K, Ohshio K, Tanaka T, Suwa M, et al. (2013) Immunomodulatory effect of Lactococcus lactis JCM5805 on human plasmacytoid dendritic cells. Clin Immunol 149: 509–518. doi: 10.1016/j.clim.2013.10.007 PMID: 24238938

19. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, et al. (2006) Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441: 101–105. PMID: 16625202

20. Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, et al. (2008) Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med 205: 1601–1610. doi: 10.1084/jem.20080091 PMID: 18591409

21. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, et al. (2006) 5'-Triphosphate RNA is the ligand for RIG-I. Science 314: 994–997. PMID: 17038590

22. Kawai T, Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 21: 317–337. doi: 10.1093/intimm/dxp017 PMID: 19246554

23. Parker JC, Whitefan MD, Richter CB (1978) Susceptibility of inbred and outbred mouse strains to Sendai virus and prevalence of infection in laboratory rodents. Infect Immun 19: 123–130. PMID: 203530

24. Yu D, Shioda T, Kato A, Hasan MK, Sakai Y, et al. (1997) Sendai virus-based expression of HIV-1 gp120: reinforcement by the V(-) version. Genes Cells 2: 457–466. PMID: 9366551

25. Kato A, Kiyotani K, Sakai Y, Yoshida T, Nagai Y (1997) The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. EMBO J 16: 578–587. PMID: 9034340

26. Pott J, Mahlakov T, Mordstein M, Duerr CU, Michiels T, et al. (2011) IFN-lambda determines the intestinal epithelial antiviral host defense. Proc Natl Acad Sci U S A 108: 7944–7949. doi: 10.1073/pnas.1100552108 PMID: 21518980

27. Saitoh T, Satoh T, Yamamoto N, Uematsu S, Takeuchi O, et al. (2011) Antiviral protein Viperin promotes Toll-like receptor 7- and Toll-like receptor 9-mediated type I interferon production in plasmacytoid dendritic cells. Immunity 34: 352–363. doi: 10.1016/j.immuni.2011.03.010 PMID: 21435386

28. Ichinohe T, Lee HK, Ogura Y, Flavell R, Iwasaki A (2009) Inflammasome recognition of influenza virus is essential for adaptive immune responses. J Exp Med 206: 79–87. doi: 10.1084/jem.20081667 PMID: 19139171

29. Asselin-Paturel C, Boonstra A, Dalod M, Durand I, Yessaad N, et al. (2001) Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol 2: 1144–1150. PMID: 11713464

30. Chaperot L, Blum A, Manches O, Lui G, Angel J, et al. (2006) Virus or TLR agonists induce TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells. J Immunol 176: 248–255. PMID: 16365416

31. Hardy AW, Graham DR, Shearer GM, Herbeuval JP (2007) HIV turns plasmacytoid dendritic cells (pDC) into TRAIL-expressing killer pDC and down-regulates HIV coreceptors by Toll-like receptor 7-induced IFN-alpha. Proc Natl Acad Sci U S A 104: 17453–17458. PMID: 17955995

32. Swiecki M, Gillillan S, Vermi W, Wang Y, Colonna M (2010) Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8+ T cell accrual. Immunity 33: 955–966. doi: 10.1016/j.immuni.2010.11.020 PMID: 21130004

33. Hase K, Kawano K, Nochi T, Pontes GS, Fukuda S, et al. (2009) Uptake through glycoprotein 2 of FirmH+ bacteria by M cells initiates mucosal immune response. Nature 462: 226–230. doi: 10.1038/nature08529 PMID: 19907495

34. Kotani Y, Kunisawa J, Suzuki Y, Sato I, Saito T, et al. (2014) Role of Lactobacillus pentosus Strain b240 and the Toll-like Receptor 2 Axis in Peyer’s Patch Dendritic Cell-Mediated Immunoglobulin A Enhancement. PLoS One 9: e91857. doi:10.1371/journal.pone.0091857 PMID: 24632732

35. Ichikawa S, Miyake M, Fujii R, Konishi Y (2009) Orally administered Lactobacillus paracasei KW3110 protects from Parainfluenza Infection. J Clin Microbiol 47: 105–110. doi:10.1128/JCM.01216-08 PMID: 19246551

36. Jang MH, Kweon MN, Iwatani K, Yamamoto M, Terahara K, et al. (2004) Intestinal villous M cells: an antigen entry site in the mucosal epithelium. Proc Natl Acad Sci USA 101: 6110–6115. PMID: 15071180

37. Rescigno M, Urbano M, Valzasina B, Francescoli M, Rotta G, et al. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. Nat Immunol 2: 1144–1150. PMID: 11713464

38. Niess JH, Brand S, Gu X, Landsman L, Jung S, et al. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 307: 254–258. PMID: 15635040

39. Kelsall BL, Leon F (2005) Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. Immunity Rev. 206: 132–148. PMID: 16048546

40. Bilsborough J, George TC, Norment A, Viney JL (2003) Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. Immunology 108: 481–492. PMID: 12667210
41. Lenschow DJ, Lai C, Frias-Staheli N, Giannakopoulos NV, Lutz A, et al. (2007) IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. Proc Natl Acad Sci U S A 104: 1371–1376. PMID: 17227866

42. Kristiansen H, Gad HH, Eskildsen-Larsen S, Despres P, Hartmann R (2011) The oligoadenylate synthetase family: an ancient protein family with multiple antiviral activities. J Interferon Cytokine Res 31: 41–47. doi: 10.1089/jir.2010.0107 PMID: 21142819

43. Kakuta S, Shibata S, Iwakura Y (2002) Genomic structure of the mouse 2',5'-oligoadenylate synthetase gene family. J Interferon Cytokine Res 22: 981–993. PMID: 12396720

44. Fitzgerald KA (2011) The interferon inducible gene: Viperin. J Interferon Cytokine Res 31: 131–135. doi: 10.1089/jir.2010.0127 PMID: 21142818

45. Jiang D, Guo H, Xu C, Chang J, Gu B, et al. (2008) Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. J Virol 82: 1665–1678. PMID: 18077728

46. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, et al. (2011) Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A 108: 5354–5359. doi: 10.1073/pnas.1019378108 PMID: 21402903

47. Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, et al. (2012) Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity 37: 158–170. doi: 10.1016/j.immuni.2012.04.011 PMID: 22705104

48. Cavanagh LL, Von Andrian UH (2002) Travellers in many guises: the origins and destinations of dendritic cells. Immunol Cell Biol 80: 448–462. PMID: 12225381