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Time-dependent persistence of enhanced immune response by a potential probiotic strain *Lactobacillus paracasei* subsp. *paracasei* NTU 101

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ABSTRACT

The possible time-dependent role of lactic acid bacteria (LAB) in immunomodulation was investigated in BALB/c mice fed daily with *Lactobacillus paracasei* subsp. *paracasei* NTU 101 (10^8 colony forming units) for 3, 6, and 9 weeks, and following feeding with *Lactobacillus*-free food for a further 7 days. We observed up-regulation of the antigen-presenting ability of dendritic cells, and expression of natural killer group-2 D (NKG2D) molecules capable of trigger natural killer cell-mediated cytotoxicity. Lymphocyte proliferation and antibody production were also significantly increased in mice after treatment. Innate and adaptive immunity remained constant even at the most protracted feeding time, indicative of the time dependence of the bacterial-mediated enhanced immunity. To better correlate intestinal microflora with immunity, the intestinal contents of probiotics and harmful microorganisms were determined. Results showed an altered intestinal microflora, with increases in bifidobacteria and lactobacilli and a decreased content of *Clostridium perfringens* after feeding with *L. paracasei* subsp. *paracasei* NTU 101. It is possible that persistent activation of immunity might be induced by intestinal probiotics.

1. Introduction

Lactic acid bacteria (LAB) are a major component of the commensal microbial flora of the human gastrointestinal tract and are frequently used as probiotics for fermentation of food products. Dietary supplementation with live beneficial bacteria promotes health and reduces the risk of various diseases (Ahrne et al., 1998). Lactobacilli play important roles in enhancement of immunity, maintenance of intestinal microbial balance and prevention of gastrointestinal infection. Immunomodulation is a very important factor contributing to anti-inflammatory, anti-infection and anti-tumor effects. It has been proposed that lactobacilli can be used for immune-stimulation to increase early lines of defense against invading pathogens (Goldin, 1998).

However, the mechanisms of immunomodulation are still unknown. It has been demonstrated that the cell wall of *Lactobacillus* contains immunomodulatory components such as polysaccharide, peptidoglycan and bacteriocin, which may be influential in activating immune responses (Meydani and Ha, 2000; Cotter et al., 2005). In murine models, several lactobacilli strains enhance both innate and adaptive immune response through the induction of dendritic cell (DC) maturation and further stimulate immune cells to release pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-12 (IL-12) (Perdigon et al., 1999). Previous studies demonstrated that different lactobacilli strain and dosage may cause different immune response (Gill et al., 2000; Maassen et al., 2000), but the correlation between immunomodulorty and lactobacilli remains unclear.

The innate immune response serves not only as the first line of defense but also has a crucial role in the development of subsequent adaptive immune responses. Mechanisms of innate immunity include enhancement of antigen presentation, phagocytosis on antigen presenting cells (APCs) and cytotoxicity by natural killer (NK) cells, which can all lead to killing of transformed cells in a seemingly non-specific fashion (Aderem and Underhill, 1999). Macrophages phagocytose the infected cells and then present antigens (Ags) to T and B lymphocytes, preluding the production of antibodies, cytokines and chemokines to modulate immune responses (Morrissette et al., 1999). Recent studies have shown *in vitro* exposure of bone marrow-derived DC to *Lactobacillus* can up-regulate surface MHC class II, B7-1 (CD80) and B7-2 (CD86) on DCs, and skew T cells from T helper 2 (Th2) toward Th1 responses, promoting humoral and cell mediated immunity (Mohamadzadeh et al., 2005). NK cells are specialized lymphocytes of the innate immune system capable of attacking virus-infected cells or transformed tumor cells via NKG2D triggered NK cell-mediated cytotoxicity (Diefenbach et al., 2000). In a previous study, *Lactobacillus casei* strain Shirota (LcS) increased NK cell activity was observed in mice and humans after ingestion of fermented milk (Hori et al., 2003; Takeda and Okumura, 2007).

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In our previous studies, *Lactobacillus paracasei* subsp. *paracasei* NTU 101 was isolated from human infant feces. Isolates display the hallmark features of good survival at low pH, tolerance to high bile concentration (Pan et al., 2002). Furthermore, the *L. paracasei* subsp. *paracasei* NTU 101-fermented milk could change of lactic acid production, sensory characteristics and ability to reduce serum cholesterol in hypercholesterolemic rats (Lin et al., 2004; Chiu et al., 2006). We have demonstrated that supplementation of human diets with *L. paracasei* subsp. *paracasei* NTU 101 may help for health benefit, but the effects of *Lactobacillus* on immune response were remain unclear. Therefore, the objective of the present study was to evaluate the mechanism of enhancing immune response by applying *Lactobacillus* for differing times using a murine model. The results demonstrate that supplementation of the diet with *L. paracasei* subsp. *paracasei* NTU 101 can persistently enhance antigen presenting ability, cytotoxicity activity, lymphocyte proliferation and antibody production, even following termination of bacteria-supplemented diet. Furthermore, the persistence activities can be induced by the increase of intestinal probiotics.

2. Materials and methods

2.1. Bacteria and growth conditions

L. paracasei subsp. *paracasei* NTU 101 was inoculated in MRS broth (BD Biosciences, San Jose, CA, USA) and grown under anaerobic conditions using an atmosphere generation system (Oxoid, Basingstoke, Hampshire, England) at 37 °C for 48 h. Thereafter, bacteria were resuspended in MRS broth to a final concentration of 1×10^9 CFU (colony-forming unit)/mL. The number and viability of the lactobacilli were determined by anaerobic cultivation on MRS plates (BD Biosciences) for 48 h.

2.2. Experimental animals

Specific-pathogen-free (SPF), male, 6–8 weeks old BALB/c mice or Sprague-Dawley rats were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Animal experiments were conducted in accordance with regulations in the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. NIH 85-23, revised 1996). Animals were provided with water and Labdiet 5001 chow (PMI Nutrition International, St. Louis, MO, USA) *ad libitum*. Animals were housed in a plastic cage and kept under pathogen-free conditions at 22±1 °C and humidity of 55±2% with a 12 h alternating light-dark cycle.

2.3. Evaluation of immune response on splenocytes

Two experiments were performed. In the first experiment, BALB/c mice were divided into seven groups. All groups received 10^9 CFU/mL *L. paracasei* subsp. *paracasei* NTU 101 in 0.1 mL MRS broth by oral administration once a day. Four groups received the bacteria for 0, 3, 6 and 9 consecutive weeks. The remaining three groups were fed for 3, 6 and 9 weeks, prior to feeding with bacteria-free chow for 7 days (3W-7d, 6W-7d and 9W-7d, respectively). Control groups received the same volume of sterile MRS broth at the same times. All data were from three independent experiments. After the final treatment, the mice were sacrificed, the spleen was removed and placed in a Petri dish, and then washed to flush out the leucocytes. The leukocytes were dispersed using a phosphate buffer saline (PBS)-filled syringe equipped with a 23-G needle. Residual red blood cells were resuspended in ammonium-chloride (ACK) lysis buffer (Tris–NH_4Cl). The intact leukocytes were recovered by centrifugation at 500 ×*g* for 5 min. After washing twice in sterile PBS, cell viability was determined by the exclusion of Trypan Blue (BD Biosciences). Viability exceeded 95% of all cell populations in all cases. Furthermore, the antigen presenting ability, NK cytotoxicity, phagocytosis of mononuclear, lymphocyte proliferation and antibody production were analyzed by fluorescence-activated cell sorting (FACS) flow cytometery (FACS calibrator; BD Biosciences).

2.4. Immunocytostaining and flow cytometry

Isolated cells were washing in sterile PBS and recovered by centrifugation at 500 ×*g* for 5 min. Add 100 μL of 5 μg/mL rat anti-mouse CD16/CD32 antibody ($2.4G_2$, Mouse Fc Block™; BD Biosciences) in PBS for 30 min on ice to reduce nonspecific antibody binding by Fc receptors. Cells were then incubated with 100 μL of 5% color-conjugated monoclonal antibody and primary monoclonal antibody, followed by immunoglobulin G (IgG)-fluorochrome isothiocyanate (FITC) at 4 °C for 30 min. Three-color immunolabeling was performed using FITC-, phycoerythrin (PE)-, allophycocyanine (APC)-, and Cychrome (Cy)-conjugated monoclonal antibody and appropriate isotype controls (Serotec, San Diego, CA, USA). The immunolabeling was used for characterization of DCs, macrophage and NK cells as follows: anti-CD11c-FITC, anti-CD49b/Pan-NK-FITC, anti-I-A/I-E (MHC II)-PE, anti-NKG2D (natural killer group-2 D)-PE, anti-mouse CD80-APC, anti-CD11b-Cy5 and anti-CD86-Cy5. During all work, cells were kept at 4 °C and at low light exposure. After washing three times with PBS, cells were applied to a FACS flow cytometer and the data were collected and analyzed using CellQuest Software (BD Biosciences). The analysis was based on counting 20,000 cells.

2.5. Preparation of crude L. paracasei subsp. paracasei NTU 101 antigen

L. paracasei subsp. *paracasei* NTU 101 was cultured with MRS broth at 37 °C for 48 h. The cultures were centrifuged at 5,000× *g* for 10 min at 4 °C, and the pellet was lysed in a lysis buffer [350 mM Tris–HCl, 10 mM EDTA (ethylene diamine tetraacetate; Sigma-Aldrich, St. Louis, MO, USA), and 1 mM PMSF (phenylmethanesulfonyl fluoride; Sigma-Aldrich)]. This solution was then sonicated on ice four times for 3 min (30% cycle, model 250; Branson Ultrasonics, Danbury, CT, USA) and centrifuged for 10 min at 12,000 ×*g* at 4 °C. The protein concentration was determined by the Bradford assay (BioRad, Munich, Germany) using bovine serum albumin (Sigma-Aldrich) as a standard, and the crude antigen was stored at −20 °C until further use.

2.6. Lymphocyte proliferation analysis

Bromodeoxyuridine (BrdU) incorporation was used to measure cell growth and DNA synthesis. Splenocytes (2×10^6 cells/mL) exposed or not exposed to 10 μg mL^{-1} or 100 μg mL^{-1} *L. paracasei* subsp. *paracasei* NTU 101 Ag were seeded in 24-well tissue culture microplates (Orange Scientific, Belgium). At 18 h before the end of the incubation period (2 days in 5% CO_2 in a humidified 37 °C incubator), 300 μM of BrdU (Sigma-Aldrich) was added per well. After incubation, the cells were then washed twice in PBS and centrifuged at 500× g for 5 min at 4 °C. Cells were incubated in 50 μL PBS containing 5 μg/mL FITC-conjugated Anti-BrdU Ab (BD Biosciences) for 30 min at 4 °C. After washing in PBS, cells were analyzed by flow cytometry as described above. Green fluorescence from the fluorescein FITC-antibody conjugate was considered a measure of BrdU incorporation. The results were expressed as the percentage of BrdU positive cells. The stimulation index (SI) was calculated by the following formula: SI=% BrdU in experimental cultures/% BrdU in control cultures.

2.7. Determination of antibody production

Blood samples were collected at different times and centrifuged to separate the sera for antibody assays. Individual serum samples were stored at −20 °C until use. IgG concentration in serum of BALB/c mice was determined by an indirect enzyme-linked immunosorbent assay (ELISA). The diluted serum (1:200) was added to each well of

microtiter plates in 100 μL aliquots. These plates were incubated 30 min at 37 °C. After washing three times with PBS containing 0.05% Tween-20 (PBST), 0.5% non-fat skim milk in PBS was added for 30 min at 37 °C to block unoccupied sites. After blocking, each plate was washed three times with PBST prior to the addition of 100 μL a 1:1000 dilution of horseradish peroxidase-conjugated goat-anti-mouse IgG (Zymed, Carlsbad, CA, USA) into each well. After incubation for 30 min at 37 °C, each plate was washed with PBST four times and each well received 100 μL of ABTS (2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) peroxidase substrate solution (Zymed) for 30 min at 37 °C. Optical density (OD) was measured at 405 nm in a PowerWave X340 microplate reader (Bio-Tek, Winooski, VT, USA).

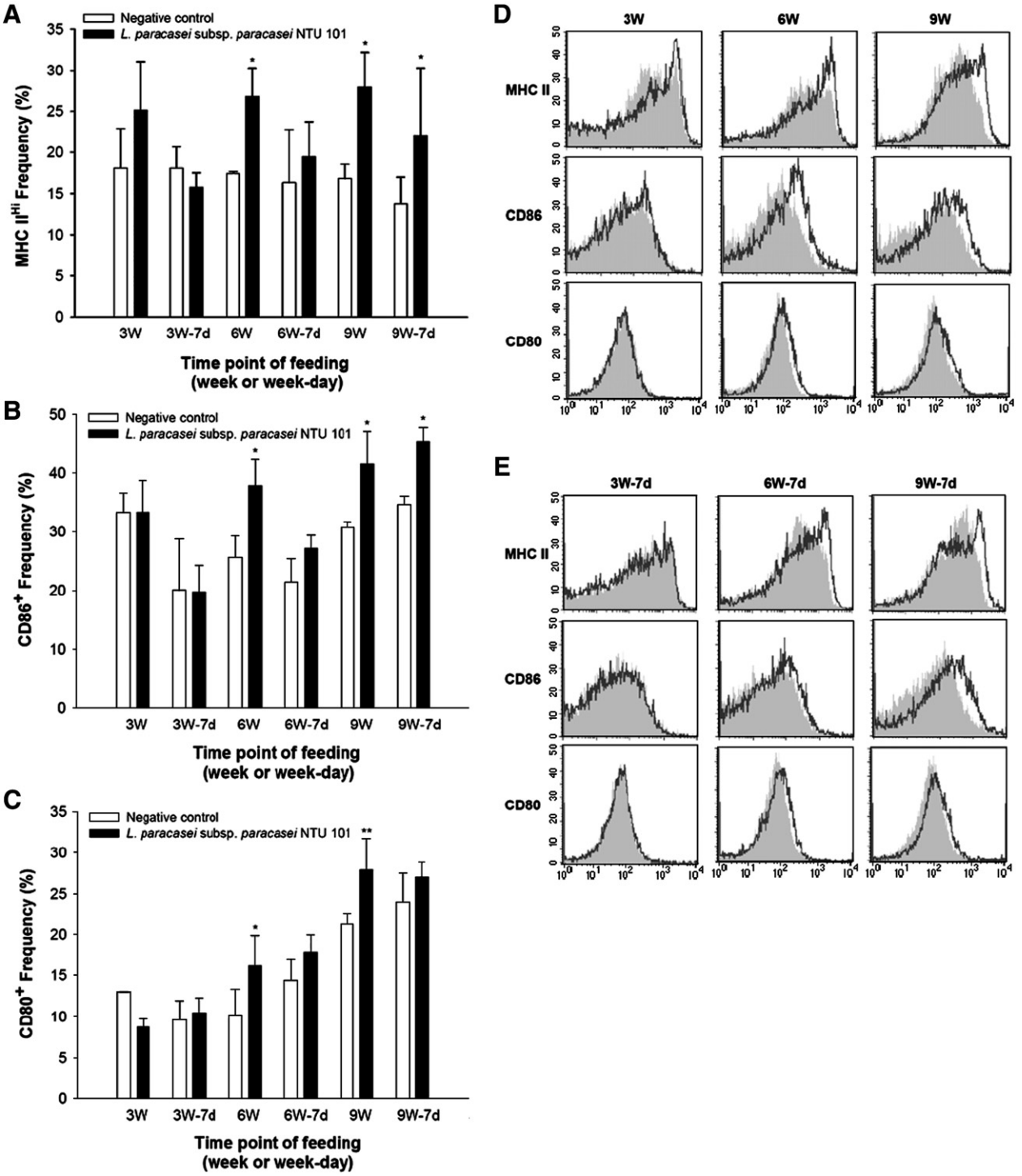

Fig. 1. FACS analysis of DCs from BALB/c mice at different feeding times after oral administration of *L. paracasei* subsp. *paracasei* NTU 101. Splenocytes were harvested, washed extensively with PBS, stained with specific Abs for 30 min at 4 °C, and analyzed by FACS. The 3W, 6W, and 9W were presented as molecular expression after oral administration for 3, 6 and 9 weeks, respectively. The 3W-7d, 6W-7d, and 9W-7d were presented as molecular expression after oral administration for 3, 6 and 9 weeks and then terminated for 7 days, respectively. The data shown are representative of typical results. All data were from three independent experiments. A–C: Results of MHC II^{hi}, CD86 and CD80 expression on DCs at different time points. The experiment group is depicted in black and the control group is depicted in white. All data are presented as mean ± S.D. (n = 6). * and ** represent significantly different from negative control ($p<0.05$ and $p<0.01$). D and E: Histograms representing flow cytometry results of expression of MHC class II, CD86 and CD80 from spleen DCs. The experiment group is depicted in black line and the control group is depicted in gray shade.

2.8. Analysis of intestinal microflora

In the second experiment, Sprague-Dawley rats were divided into four groups. They received 10^9 CFU/mL *L. paracasei* subsp. *paracasei* NTU 101 in 0.1 mL MRS broth or bacteria-free MRS broth by oral administration once a day for 3, 6 and 9 consecutive weeks. All data were from three independent experiments. After the different times, the feces of each Sprague-Dawley rat was collected and placed in a capped test tube, which was taken to a lamina flow cabinet where 1 g of each feces sample was weighed, transferred into a tube with 9 mL of anaerobic diluents, and homogenized by vortexing. The same procedure was repeated several times to perform a serial dilution. The bacterial viability was determined by determining CFUs after plating on MRS (lactobacilli), BIM-25 (bifidobacteria) and TSC w/egg yolk agar (*Clostridium perfringens*) (BD Biosciences). The plates were placed in anaerobic containers at 37 °C for 48 h.

2.9. Statistical analysis

Values shown represent the mean±SD of separate experiments. Data were analyzed using the one-way ANOVA procedure of SPSS software (Cary, NC, USA). The differences among means were detected by the Duncan's Multiple Range Test. Data were considered significantly different ($p<0.05$) in variables between groups.

3. Results

3.1. L. paracasei subsp. paracasei NTU 101 up-regulate maturation of DC surface marker expression

To investigate surface marker expression on DCs at different time after administration with *L. paracasei* subsp. *paracasei* NTU 101, flow cytometry analysis was performed. Upon isolating splenocytes from mice, cells were examined by flow cytometry using the DC marker CD11c. Based on the level of MHC class II expression on the cell surface, immature and mature DC subsets can be recognized as MHC class II^{low} and MHC class II^{hi} DC, respectively (Lutz et al., 1999). The expression of MHC was then evaluated by MHC class II^{hi}. The data showed that MHC class II^{hi} were activated by *L. paracasei* subsp. *paracasei* NTU 101 at 6 and 9 weeks (Fig. 1A and D). The level of MHC class II^{hi} activation was significantly different from negative control ($p<0.05$). Interestingly, the activation of MHC class II^{hi} was 112.5% greater than the negative control in 9W-7d group ($p<0.05$) (Fig. 1E). Similar results of MHC class II molecule expression on macrophages were also observed (data not shown).

The expression of co-stimulatory molecules CD86 (B7.2) and CD80 (B7.1) activation were significantly different from that of negative control after administration for 6 weeks ($p<0.05$) (Fig. 1B and C). In the 6W-7d group, activation of CD80 and CD86 were increased 26.5%

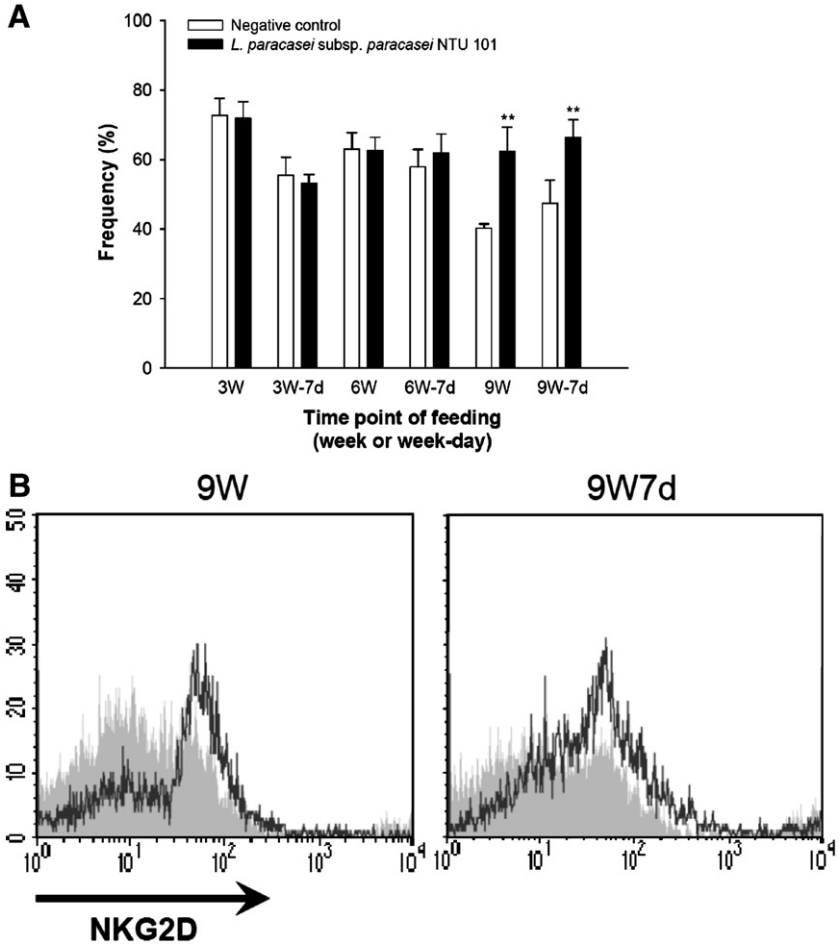

Fig. 2. Expression of NKG2D at different feeding times on spleen NK cell from BALB/c mice after orally administered with *L. paracasei* subsp. *paracasei* NTU 101. Splenocytes were harvested, washed extensively with PBS, stained with specific Abs for 30 min at 4 °C, and analyzed by FACS. The 3W, 6W, and 9W were presented as molecular expression after oral administration for 3, 6 and 9 weeks, respectively. The 3W-7d, 6W-7d, and 9W-7d were presented as molecular expression after oral administration for 3, 6 and 9 weeks and then terminated for 7 days, respectively. The data shown are representative of typical results. All data were from three independent experiments. (A) Results of NKG2D expression on NK cells at different time points. The experiment group is depicted in black and the control group is depicted in white. All data are presented as mean±S.D. ($n=6$). ** represent significantly difference from negative control ($p<0.01$). (B) Histograms representing flow cytometry results of expression of NKG2D from spleen NK cells. The experiment group is depicted in black line and the control group is depicted in gray shade.

and 24.3%, respectively, relative to the negative control, but only CD86 molecule activation was constantly expression even through the 7 day bacteria-free diet following the 9 week administration of lactobacilli ($p<0.05$) (Fig. 1E). These results indicated *L. paracasei* subsp. *paracasei* NTU 101 could enhance antigen presenting ability on DCs, and that the persistence of the surface marker was time-dependent.

3.2. Enhancement of NKG2D triggers NK cell-mediated cytotoxicity by *L. paracasei subsp. paracasei* NTU 101

Previous studies have shown that NKG2D receptor can be constitutively expressed on NK cell, and that the NKG2D–DAP-10 complex can trigger cytotoxicity accompanied with killing of tumor cells following NKG2D cross-linking in NK cells (Coudert and Held, 2006). To determine whether activation of cytotoxicity via NKG2D-dependent NK cells is mediated by *L. paracasei* subsp. *paracasei* NTU 101, splenocytes from mice were verified by flow cytometry using the NK cell marker NK1.1. NK cell numbers in splenocytes were not different among any of the time intervals (data not shown). As seen in Fig. 2A, NKG2D expression decreased after 9 weeks in control mice. But the level of activation was significantly increased after oral administration of *L. paracasei* subsp. *paracasei* NTU 101 for 9 weeks ($p<0.01$). Moreover, the activation of NKG2D was constantly expressed in the 9W-7d group ($p<0.01$, Fig. 2B). These observations indicated that *L. paracasei* subsp. *paracasei* NTU 101 could up-regulate cytotoxicity via NKG2D-dependent pathway and that the persistent expression was time-dependent.

3.3. *L. paracasei subsp. paracasei* NTU 101-induced proliferation of spleen lymphocytes

According to our previous experiments, DC activation was induced by *L. paracasei* subsp. *paracasei* NTU 101 after administration for 6 weeks. Based on the knowledge that DCs activate the adaptive immune response by priming lymphocyte to proliferate and produce antibody, the influence of *Lactobacillus* on lymphocyte proliferation was measured. After isolation of splenocytes, the cells were incubated with *L. paracasei* subsp. *paracasei* NTU 101 Ag at different time intervals. The effect of immunostimulation was evaluated by stimulation index. As shown in Table 1, splenocytes from control mice showed spontaneous proliferation at different time intervals. However, the proliferation levels of splenocytes from treated mice were significantly higher than those of control mice after administration for 9 weeks ($p<0.05$). Similarly, strong proliferation was evident in the 9W-7d group ($p<0.05$), whereas no difference was found among other time intervals after feeding *Lactobacillus*. These results suggested that lymphocyte proliferation affected by *L. paracasei* subsp. *paracasei* NTU 101 was time-dependent.

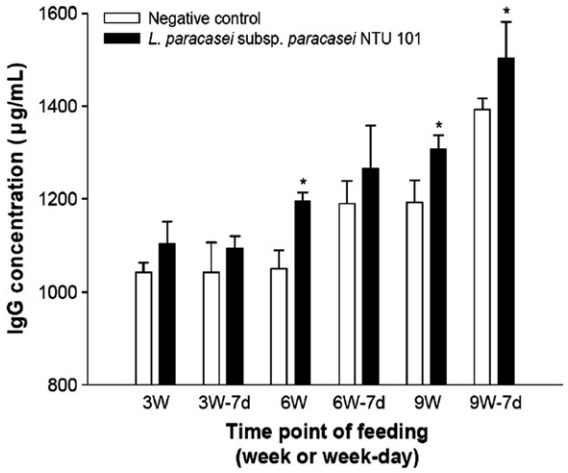

Fig. 3. Production of IgG antibodies at different feeding times from BALB/c mice after orally administered with *L. paracasei* subsp. *paracasei* NTU 101. All data are presented as mean±S.D. ($n=6$). The experiment group is depicted in black and the control group is depicted in white. * represents significantly different from negative control ($p<0.05$). The 3W, 6W and 9W represent antibody concentration change after oral administration for 3, 6 and 9 weeks. The 3W-7d, 6W-7d and 9W-7d represent antibody concentration change after oral administration for 3, 6 and 9 weeks and then termination for 7 days. All data were from three independent experiments.

3.4. Production of serum IgG antibody after *L. paracasei subsp. paracasei* NTU 101 administration

Following Ag recognition by Ag-presenting cells, B cells become activated and differentiated into antibody-secreting plasma cells to produce IgG antibodies, a most important antibody of adaptive immunity (Ollila and Vihinen, 2005). Therefore, we examined the effects of *Lactobacillus* on the IgG level in mice serum by sandwich ELISA. The data showed that after feeding mice with *L. paracasei* subsp. *paracasei* NTU 101 for 6 or 9 weeks significantly increased IgG antibodies in serum compared with control mice ($p<0.05$). The increased concentration of IgG antibodies was maintained in the 9W-7d group ($p<0.05$, Fig. 3), but not in the 3W-7d and 6W-7d groups, indicative of the time-dependence of the effect.

3.5. *L. paracasei subsp. paracasei* NTU 101 regulation of microflora in the intestinal tract

In previews experiments, the expression of innate and adaptive immunity in mice was enhanced by *L. paracasei* subsp. *paracasei* NTU 101 and the activation of immune cells was time-dependent. To better understand the possible mechanism of the enhanced immune response, we sought to determine whether intestinal microflora

Table 1
Effect of *L. paracasei* subsp. *paracasei* NTU 101 administration to mice on slpeen lymphocyte proliferation under stimulation with homologous antigen preparation

Feeding time[a]	Control					Experiment				
	Non-treated[b]	1 ×NTU101		10×NTU101		Non-treated	1×NTU101		10×NTU101	
			SI[c]		SI			SI		SI
3W	4.52±0.26	5.31±0.18	1.18	5.67±0.49	1.25	4.62±0.40	5.56±0.37	1.20	6.25±0.41	1.35
6W	5.41±1.42	6.70±1.05	1.24	10.29±1.31	1.90	6.03±0.77	8.74±0.56	1.45	13.74±2.51	2.28
9W	6.45±0.35	11.38±1.01	1.76	18.32±3.60	2.84	7.77±0.97	20.46±0.59[d]	2.63	29.85±3.33[d]	3.84
3W-7d	4.81±0.41	5.96±0.35	1.24	6.01±0.49	1.25	5.07±0.24	6.78±0.41	1.34	6.96±0.22	1.37
6W-7d	6.52±0.41	7.97±0.66	1.22	10.52±1.92	1.61	7.05±0.24	8.77±0.37	1.24	12.39±1.47	1.76
9W-7d	6.62±0.14	8.21±0.98	1.24	17.22±1.08	2.60	6.86±0.43	11.49±2.17[d]	1.67	24.03±2.91[d]	3.50

All data from three independent experiments are presented as mean±S.D. ($n=6$).
The control group was fed with MRS broth only, and the experiment group was fed with 10^8 CFU of *L. paracasei* subsp. *paracasei* NTU 101/daily for different times.
[a] The 3W, 6W and 9W represent oral administration for 3, 6, and 9 weeks. The 3W-7d, 6W-7d, and 9W-7d were presented after oral administration for 3, 6, and 9 weeks and then termination for 7 days.
[b] FACS analysis after stimulation without (non-treated) or with 10 μg mL^{-1} (1×) and 100 μg mL^{-1} (10×) NTU 101 antigen for 2 days.
[c] SI was calculated by the following formula: SI=% BrdU in experimental cultures/% BrdU in control cultures.
[d] Significant difference ($p<0.05$) from the cells of control mice with the same dose of antigen stimulation.

Table 2
Quantitative analysis of intestinal microflora at different times after administration of *L. paracasei* subsp. *paracasei* NTU 101

	Group	Feeding time ($\log_{10}$ CFU/g)			
		0	3	6	9
Bifidobacterium spp	Control	6.75±0.61	6.75±0.39	6.37±0.26	6.87±0.32
	Experiment	6.86±0.34	7.11±0.54	6.86±0.49*	7.35±0.62*
Lactobacillus spp	Control	7.47±0.42	7.63±0.18	7.32±0.40	7.49±0.17
	Experiment	7.35±0.63	7.73±0.22	7.19±0.26	7.85±0.27*
C. *perfringens*	Control	3.19±0.41	3.22±0.56	3.98±0.52	2.66±0.61
	Experiment	2.98±0.88	3.21±0.54	3.24±0.50*	2.15±0.47*

All data from three independent experiments are presented as mean±S.D. ($n=8$).
The control group was fed with MRS broth only, and the experiment group was fed with 10^8 CFU of *L. paracasei* subsp. *paracasei* NTU 101/daily for different times.
*Significantly different from control group ($p<0.05$).

were involved. We determined the bacterial counts in the feces at different time intervals. As shown in Table 2, bifidobacteria and lactobacilli counts were significantly increased after feeding for 6 or 9 weeks, respectively ($p<0.05$). Furthermore, *C. perfringens* was markedly decreased after feeding for 6 and 9 weeks compared with control mice ($p<0.05$).

4. Discussion

Many *Lactobacillus* species regulate the innate and adaptive immune responses, including modulated expression of cytokines, maturation of surface markers in immune cells, lymphocyte responses or antibody production (He et al., 2005). Lactobacilli are potent inducers of Th1-type cytokines such as IL-2, IFN-γ, and TNF-α in a dose-dependent manner, but not of Th2 cytokines (Hessle et al., 1999). Although previous studies have established that lactobacilli are able to enhance immunity in mice, and this effect is dose- and strain-dependent (Paturi et al., 2007), correlation between administration time and regulation of immunity by *Lactobacillus* remains unclear. Therefore, the interaction of *L. paracasei* subsp. *paracasei* NTU 101 and immunity was investigated in the present study.

Initiation of the immune response requires activation of MHC class II and co-stimulatory signals in the form of CD80 and CD86 molecules provided by professional antigen presenting cells (APC) (Harris and Ronchese, 1999). Previous study has established that DC maturation is up-regulated though the expression of C80, CD86, and MHC class II after interaction with probiotics. Moreover, the regulation of DC cytokines by probiotics may contribute to the benefit in treating the intestinal diseases (Drakes et al., 2004). Therefore, DCs play a crucial immunoregulatory role in immune responses under antigen uptake and processing such as the induction of Ag-specific immune responses and Th1/Th2 balance. Different *Lactobacillus* species cause distinct expression of MHC class II, CD80 and CD86 molecules on DCs (Christensen et al., 2002). In this study, we also observed up-regulation after administration with *L. paracasei* subsp. *paracasei* NTU 101 in 6W, 9W and 9W-7d groups. MHC class II activity on macrophage also showed a similar result. The results suggest *Lactobacillus* not only enhances APC activation in the feeding period, but also the persistence of activation under long-period treatment. DC maturation not only induces the adaptive immune response, but also supports the tumoricidal activity or early stages of viral infection on NK cells (Fernandez et al., 1999). Furthermore, *Lactobacillus* significant increases cell populations and cytotoxicity ability on NK cells in mice and human (Lee et al., 2004; Takeda and Okumura, 2007). NKG2D is one of the major activating receptors on NK cells, which mediate cytotoxic activity by recognizing defined ligands, often over-expressed on transformed cells (Cerwenka and Lanier, 2001). The abolition of tumor cell killing when NKG2D-blocking antibody is added suggests that cytotoxicity depends on NKG2D receptor function (Ostberg et al., 2007). Here, we used NKG2D to evaluated cytotoxic activity. We observed that NKG2D decreased with time, whereas expression still continue to be enhanced after administration with *L. paracasei* subsp. *paracasei* NTU 101 in the elderly mice. These data demonstrate that innate immunity, evident as APC and NK cell activity, are increased by *L. paracasei* subsp. *paracasei* NTU 101 in a time-dependent manner.

The cells of the adaptive immune system are lymphocytes. T and B cells are the major types of lymphocytes. T cells are immune response mediators, and play an important role in establishing and maximizing the capabilities of the adaptive immune response. B cells are the major cells involved in the creation of antibodies that circulate in blood plasma and lymph. Activation by APC interaction in turn spurs activation of naïve lymphocyte; their subsequent activation, proliferation and differentiation leads to the secretion of antibodies and cytokines. Previously, it was demonstrated that oral administration of *Lactobacillus* enhances specific adaptive immune responses via innate immunity in mice (Galdeano and Perdigon, 2006). In the present study, spleen cells and serum from mice fed with *L. paracasei* subsp. *paracasei* NTU 101 showed significant increases of lymphocytes and antibody production, and the expression pattern was similar to our previous DCs maturation experiment. Based on the above findings, we suggest that activation of adaptive immunity is regulated by DC activation. Furthermore, *L. paracasei* subsp. *paracasei* NTU 101 not only enhances the immunity in the feeding period, but also the persistence of activation under long-period treatment.

Probiotics beneficially affect the host by improving the balance of the intestinal flora (Fuller, 1989). Probiotic intake should lead to the creation of gut microbiology conditions that suppress harmful microorganisms (Rada and Rychly, 1995; Mountzouris et al., 2007) and favor beneficial microorganisms, ultimately enhancing gut health. In a previous study, we demonstrated the number of bifidobacteria and lactobacilli are increased in the cecum of hypercholesterolemic hamsters after feeding with *L. paracasei* subsp. *paracasei* NTU 101 (Chiu et al., 2006). To better understand the effect of intestinal microflora and immunity, we measured probiotics and harmful microorganisms in the intestinal tract at different time intervals. The results showing the altered composition of intestinal microflora support the suggestion that the persistence of immune activation may be caused by the intestinal probiotics.

In conclusion, *L. paracasei* subsp. *paracasei* NTU 101 is able to persistently enhance both innate and adaptive immune responses, e.g DC maturation, NK cell activity, lymphocyte proliferation and antibody production. It is noteworthy that the ability to stimulate immune response is time-dependent. Furthermore, the persistence of enhancing immunity may be due to the regulation of intestinal microflora, increased probiotics and suppression of harmful microorganisms. This paper, which represents the first report of the enhancement of immunity by *Lactobacillus* in time-dependent manner, raises an interesting possibility that supplementation of human diets with *L. paracasei* subsp. *paracasei* NTU 101 may convey a health benefit.

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