



A human milk oligosaccharide, 2'-fucosyllactose, enhances the immunity in mice fed an infant formula milk diet

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ABSTRACT

The immune effect of 2'-fucosyllactose (2'FL) in mice fed an infant formula (IF) milk diet was studied. Forty-five BALB/C mice were randomly divided into 3 groups: control, and low and high dose of 2'FL. 2'FL was administered once daily for 30 days and all the mice were fed IF milk diet. On day 30, the spleen and thymus were isolated and weighed for immune organ index calculation. Natural killer (NK) cell activity, T-lymphocyte proliferation capacity and delayed-type hypersensitivity reactivity (DTH) were measured. There was a significant increase in thymus index in high dose 2'FL group ($p < 0.05$). T-lymphocytes in high dose 2'FL group also tended to proliferate more compared with the control group ($p < 0.05$). NK cell activity and the degree of DTH were improved in both 2'FL groups ($p < 0.05$). These data demonstrated that 2'FL can improve immunity in mice fed an IF milk diet.

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1. Introduction

Human milk confers multiple layers of protection to the infants by providing bioactive components that help the immune development, support healthy gut microbes, etc. (Donovan & Comstock, 2016). Human milk oligosaccharides (HMOs) are the third most abundant component in human milk and are composed of linear or branched units of monosaccharides including glucose, galactose, *N*-acetylglucosamine, fucose and sialic acid (neuraminic acid) (Ninonuevo et al., 2006). Although HMO profiles produced by women vary geographically (Mcguire et al., 2017), 2'-fucosyllactose (2'FL) is still one of the most predominant HMOs in human milk (Goehring et al., 2016). As methods of the synthesis of HMOs has developed, commercially produced 2'FL is becoming increasingly available (Donovan & Comstock, 2016). In recent years, 2'FL has GRAS (Generally Recognized As Safe) approval in America (Salminen, 2017). Further, it has passed the safety assessment for applying to infant formula in European Union (Salminen, 2017). As the composition and nutrition of infant formulas should be designed to mimic human milk to supply complete nutrition for

infants as closely as possible (Chen et al., 2019), 2'FL might be a good candidate if applied to infant formula.

Immunity is the state of not being susceptible to the pathogenic effects of foreign microorganisms or to the toxic effect of antigenic substances. The immunoregulation mechanism of 2'FL has been reported. Kulinich and Liu (2016) reported that 2'FL may possess biological activities such as regulation of immune responses via influencing different cell populations. The structure of 2'FL is similar to that of selectin ligand, which is an important immune mediator and is expressed in immune cells; 2'FL can bind to immune cells and affect the immune system in the intestinal cells (Bode, 2006; Triantis, Bode, & van Neerven, 2018).

On the other hand, more evidence of immune modulation effect of 2'FL is needed. There are few human studies on the immune outcome of 2'FL (Triantis et al., 2018). Morrow et al. (2004) found that *Campylobacter* diarrhoea occurred less often in infants whose mother's milk contained high levels of 2'FL. Puccio et al. (2017) showed that infants receiving formula with 1.0 g L^{-1} 2'FL and 0.5 g L^{-1} lacto-*N*-neotetraose (LNnT) had more body weight than infants receiving non-HMO formula from 14 days to 6 months. Plasma inflammatory cytokines were reduced in 2'FL treatment group in another study (Goehring et al., 2016). In animal studies, 2'FL showed a good anti-infection effect involving in many disease models (Triantis et al., 2018). More evidence is needed to show the immune effect of 2'FL in a no-disease model. Interestingly, most

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reports on HMOs are about the effect of promoting humoral immunity (Kulinich & Liu, 2016). The effect of 2'FL on cellular immunity is hardly reported (Castillo-Courtade et al., 2015; Xiao et al., 2018); the related immune indicators were mainly focused on the cytokines and the amount of immune cells (Bode et al., 2004; Comstock, Wang, Hester, Li, & Donovan, 2014; Zehra et al., 2018).

The immunity effect of 2'FL, with special focus on the natural immunity and cellular immunity in a no-disease-model, was therefore studied in this research with mice fed an infant formula diet. The immune organ index, the innate immune function and the cellular immune function were assessed to reveal the effect of 2'FL on immune function.

2. Materials and methods

2.1. Infant formula

The infant formula milk used was a basic formula manufactured from bovine milk as the base powder and non-dairy ingredients such as vegetable oil. The milk was obtained from a pool of fresh milk from two herds of Holstein and Jersey cows at Ausnutria Dairy private farm (Changsha, China). The raw milk was sterilised at 85 °C for 15s and evaporated twice at 72 °C (39kpa) and 50 °C (68kpa), respectively. The product was then dried by spray drying. The composition of the infant formula milk comprised 11.06% protein, 26.20% fat, 53.43% carbohydrate, and 97.35% total solid.

2.2. Animals and treatment

The study design diagram is shown in Fig. 1. This research was performed in accordance with protocols approved by the Central South University Institutional Animal Care and Use Committee, China. Five grams of infant formula milk were dissolved in 30 mL cold-boiled water and presented in each cage of five mice every morning. The remaining milk volumes were recorded the next day. As infant formula milk is not the ideal food for mice, the mice were also fed chow (SJA Laboratory Animal Co., Ltd, Changsha, China) at kcal ratio of 7:3 (milk:chow) according to a former study in our laboratory (Zhou, Hang, Wang, & Fang, 2018). All the chow was

consumed every day in every cage. The composition of chow comprised 20% protein, 4% fat, 5% fibre, 8% crude ash, 2.5% total microelement and 0.4% sodium chloride.

Forty-five BALB/C male mice (7 weeks old) were randomly divided into three groups: control group that received normal saline (NS), and two groups treated with 2'FL (Seebio Biotech (Shanghai) Co., Ltd, China; purity 91.9%) at a low (1 g kg⁻¹ body weight) and high (2 g kg⁻¹ body weight) dose. The 2'FL also contained 1.7% lactose, 2.0% 3-fucosyllactose, 2.0% difucosyllactose, 1.6% fucosyl-galactose, and 0.8% fucose. The low and high doses of 2'FL were roughly equivalent to 5 and 10 times of 1% (w/w) 2'FL-supplemented infant formula, respectively. The administration of NS and 2'FL treatments by oral gavage once daily for 30 days (Thum, McNabb, Young, Cookon, & Roy, 2016). The animals were weighed every two days. Animals were housed in a specific pathogen-free room at a constant temperature (22 °C ± 1 °C) and humidity (55%) and were allowed free access to infant formula milk.

2.3. Immune organ index

On day 30, the mice killed by cervical dislocation and weight. The spleen and thymus were removed, washed, drained and weight. Then, the spleen and thymus index were both calculated (Li et al., 2018) using equations (1) and (2), respectively:

$$\text{Spleen index (mg g}^{-1}\text{)} = \frac{\text{spleen weight(mg)}}{\text{body weight(g)}} \quad (1)$$

$$\text{Thymus index (mg g}^{-1}\text{)} = \frac{\text{thymus weight(mg)}}{\text{body weight(g)}} \quad (2)$$

2.4. Natural Killer cell activity

The splenic Natural Killer (NK) cell activity was estimated by the method of Konjevic, Jurisic, and Spuzic (2001). Briefly, the spleen was excised and placed in a 200-mesh stainless steel sieve on a culture dish (Biofil, Changsha, China) containing Hank's solution (Solarbio, Changsha, China). The spleen was ground into small pieces with the plunger of a glass syringe. The suspension was

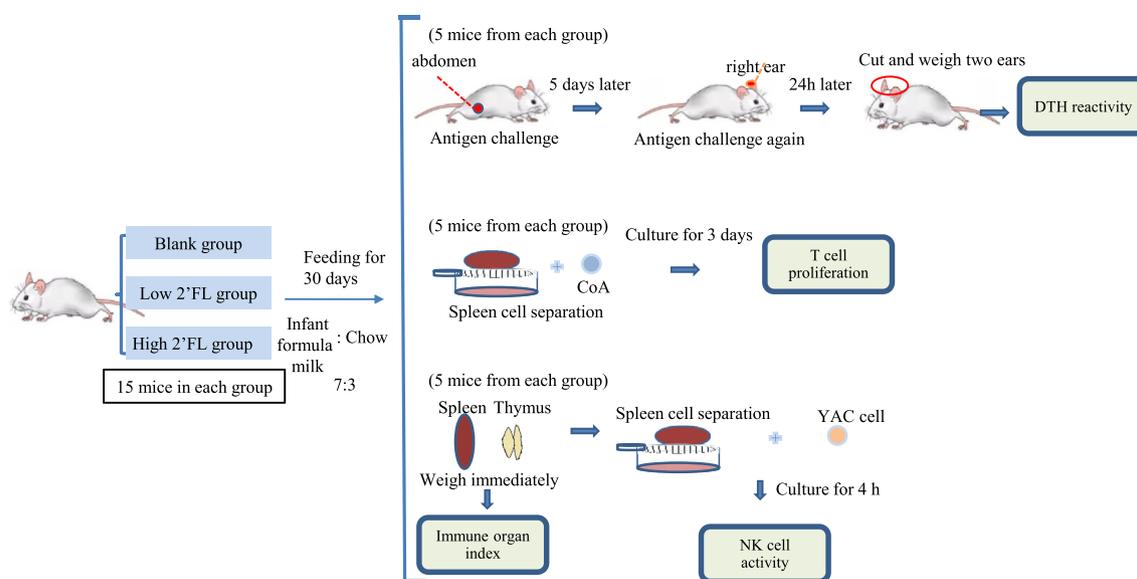


Fig. 1. Study design diagram. Abbreviations are: 2'FL, 2'-fucosyllactose; DTH, delayed-type hypersensitivity reactivity; YAC, mouse lymphoma cell; ConA, concanavalin A. Low and high levels of 2'FL were 1 and 2 g kg⁻¹ of 2'FL, respectively. The mice were all fed an infant formula milk diet for 30 days; 5 mice from each group were processed for each study measure (the same mice were used for the immune organ index and NK cell activity tests).

centrifuged ($201 \times g$, 10 min) and was adjusted at concentration of 2×10^7 cells mL^{-1} with 1640 medium (Gibco, Changsha, China). Mouse lymphoma cell (YAC-1, Shanghai Cell Bank, China) was adjusted to 4×10^5 cell mL^{-1} . Then, 100 μL YAC-1 cells were mixed with spleen cell, 1640 medium, and 2.5% Triton solution (Solarbio) as reaction well, releasing well and maximum releasing well, respectively. The experiments were performed in triplicate. The cell suspensions were then incubated for 4 h and centrifuged ($453 \times g$, 5 min). One hundred millilitres of supernatant were mixed with 100 μL lactic dehydrogenase (LDH) reaction solution [1.3×10^{-3} mol L^{-1} NAD^+ (Solarbio), 6.6×10^{-4} mol L^{-1} lodonitrotetrazolium chloride (Solarbio), 2.8×10^{-4} mol L^{-1} phenazine methosulphate (Solarbio), 5×10^{-2} mol L^{-1} sodium lactate (Mackline, Changsha, China), 0.1 mol L^{-1} PBS (Dingguo, Changsha, China)]. The optical density at 490 nm wavelength was then analysed using an enzyme-linked immunoassay absorbance reader (96 well plate method; Powerwave XS2, Biotek, Gen Group Company, USA) and NK cell activity calculated [equation (3)]:

$$\text{NK cell activity} = \frac{\text{OD in reaction well} - \text{OD in normal releasing well}}{\text{OD in max releasing well} - \text{OD in normal releasing well}} \times 100\% \quad (3)$$

2.5. T-lymphocyte proliferation

The proliferation function of splenic T lymphocyte was measured using a method based on the study of Choque Delgado, Thome, Gabriel, Tamashiro, and Pastore (2012). Briefly, the spleen cells were adjusted to a concentration of 3×10^6 cell mL^{-1} . In triplicate, 1 mL of cell suspension was pipetted into two wells of a microplate and 75 μL 100 μg mL^{-1} concanavalin A (ConA, Sigma, Changsha, China) added to one well. The cells were incubated for 68 h at which time 0.7 mL cell supernatant was discarded and 0.7 mL 1640 medium and 50 μL methyl thiazolyl tetrazolium (MTT, Genview, Changsha, China) were added to the wells and the incubation continued for a further 4 h (72 h in total). One millilitre of acid isopropanol [1 mol L^{-1} hydrochloric acid:isopropanol (both from Wokai, Changsha, China) 1:24, v/v] was then added to each well. The optical density at 570 nm was determined using an enzyme-linked immunoassay absorbance reader (24 well plate method; Powerwave XS2). The absorbance difference was calculated [equation (4)]:

$$\text{T lymphocyte proliferation capacity} = \frac{\text{OD in wells with ConA} - \text{OD in wells without ConA}}{\text{OD in wells without ConA}} \times 100\% \quad (4)$$

2.6. Delayed-type hypersensitivity reactivity in vivo

According to the method of Corsini, Silvia, and Costa (1979), 50 μL dinitrofluorobenzene (Mackline) as a modest antigen were evenly applied to the abdomen of mice after shaving to sensitise T-lymphocytes on day 30, enabling the cellular immune response to be easily induced when the body re-encountered dinitrofluorobenzene. Five days later, the treatment was carried again by

applying 10 μL dinitrofluorobenzene to the inside and outside pinnae of the right ear; 24 h later, the mice were sacrificed. The second treatment induced delayed-type hypersensitivity (DTH) reactivity, i.e., the protection of the host against the antigen. Infiltration of mononuclear cells and tissue oedema in challenged ears are typical histological changes in localised DTH reaction due to the binding interaction of sensitised T-lymphocytes and antigen. This resulted in the weight of the right ears of the mice increasing relative to the left ears. The ears in diameter of 8 mm were collected and weighed; the level of increased mass reflected the immune response intensity. If the response intensity is stronger, the immune level is higher. Ear weight increasing ratio was calculated [equation (5)]:

$$\text{Ear weight increase(mg)} = \frac{\text{right ear weight(mg)} - \text{left ear weight(mg)}}{\text{left ear weight(mg)}} \quad (5)$$

2.7. Statistical analysis

All data were analysed using SPSS statistic 18.0 software. NK cell activities were transformed by arcsine transformation. The data between groups were analysed by one-way analysis of variance (ANOVA). If there were statistical significance, Dunnett-t post hoc test were conducted. Data are presented as mean \pm standard deviation and statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Body weight and food intake

The body weight in the three groups did not show any significant difference ($p > 0.05$, $n = 15$ in each group; Fig. 2A); the average values (g) were 20.62 ± 1.60 , 21.05 ± 1.42 and 21.17 ± 1.63 , respectively). The total caloric of food intake in the three experimental groups also showed no statistical difference ($p > 0.05$, $n = 15$ in each group; Fig. 2B, Table 1). The energy from 2'FL and the

energy proportion of total food intake of 2'FL significantly increased in the two 2'FL treatment groups, compared with the control group ($p < 0.05$; Table 1).

3.2. 2'-Fucosyllactose increases the thymus index

The immune organs are important places for immune cell maturation and immune response; the thymus and spleen are key

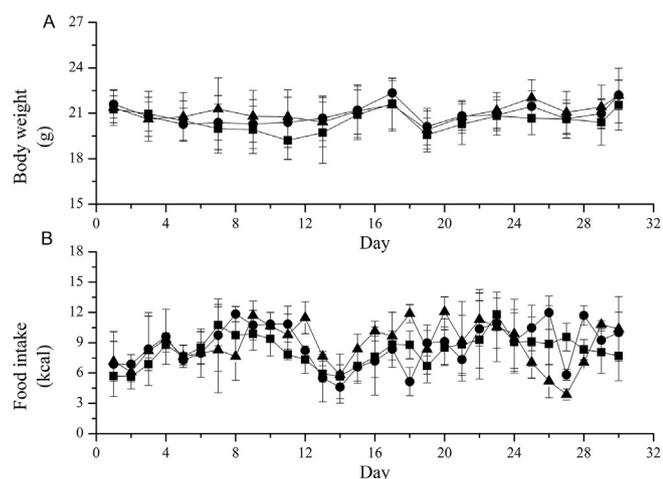


Fig. 2. Body weight (A) and food intake (B) in the three groups. The low (●) and high (▲) groups were gavaged with 1 and 2 g kg⁻¹ of 2'-fucosyllactose once daily for 30 days, respectively. The control mice (■) were gavaged with 10 g kg⁻¹ normal saline. Values are expressed as the mean ± SEM of 15 mice in each group; no significant differences were found in the three groups ($P > 0.05$).

Table 1
Food intake energy in the three experimental groups.^a

Parameter	Control	2'FL low dose	2'FL high dose
Infant formula milk and chow (kcal)	8.27 ± 2.15	8.68 ± 2.71	8.75 ± 2.70
2'FL (kcal)	0 ^a	0.046 ^b	0.092 ^c
Total caloric intake (kcal)	8.27 ± 2.15	8.72 ± 2.71	8.84 ± 2.70
2'FL/total energy (%)	0 ^a	0.59 ± 0.24 ^b	1.17 ± 0.46 ^c

^a Abbreviation: 2'FL, 2'-fucosyllactose. The low and high groups were gavaged with 1 and 2 g kg⁻¹ of 2'FL, respectively, once daily for 30 days. Values are the mean ± SEM of 15 mice in each group; values with different superscript letters in a row are significantly different ($p < 0.05$).

organs of the immune system and, to a certain extent, the spleen and thymus indices reflect the condition of immune organ function. The thymus index and the spleen index were recorded on day 30. The thymus index in high group (2 g kg⁻¹ 2'FL) revealed a significant increase compared with the control group; the average values (mg g⁻¹) in control, low and high groups were 0.30 ± 0.10, 0.48 ± 0.34 and 1.11 ± 0.22, respectively ($p < 0.05$, $n = 5$ in each group; Fig. 3A). The spleen index did not show any statistically significant changes; the average values (mg g⁻¹) in control, low and high groups were 3.93 ± 0.49, 4.05 ± 0.36 and 3.83 ± 0.31, respectively).

3.3. 2'-Fucosyllactose improves splenic NK cell activity

NK cells are important in natural immunity function. To determine the effects of 2'FL on NK cell, we used YAC-1 as the target cell and detected the activity of released LDH. Both low and high doses of 2'FL significantly improved NK cell activity compared with the control group; the average values (%) of NK cell activity in control, low and high groups were 0.83 ± 0.09, 1.08 ± 0.17 and 1.28 ± 0.21, respectively ($p < 0.05$, $n = 5$ in each group; Fig. 3B).

3.4. 2'-Fucosyllactose upregulates T lymphocyte proliferation

The ability of T lymphocyte proliferative plays an important role in cellular immunity function. The absorbance difference between wells with and without ConA reflected the proliferative capacity of T cells. In contrast to the low dose of 2'FL that had no effect, the high dose of 2'FL significantly increased proliferation of spleen lymphocytes; the average values (%) of T lymphocyte proliferative capacity in control, low and high groups were 25.70 ± 8.48, 40.72 ± 9.26 and 60.60 ± 9.04, respectively ($p < 0.05$, $n = 5$ in each group; Fig. 3C).

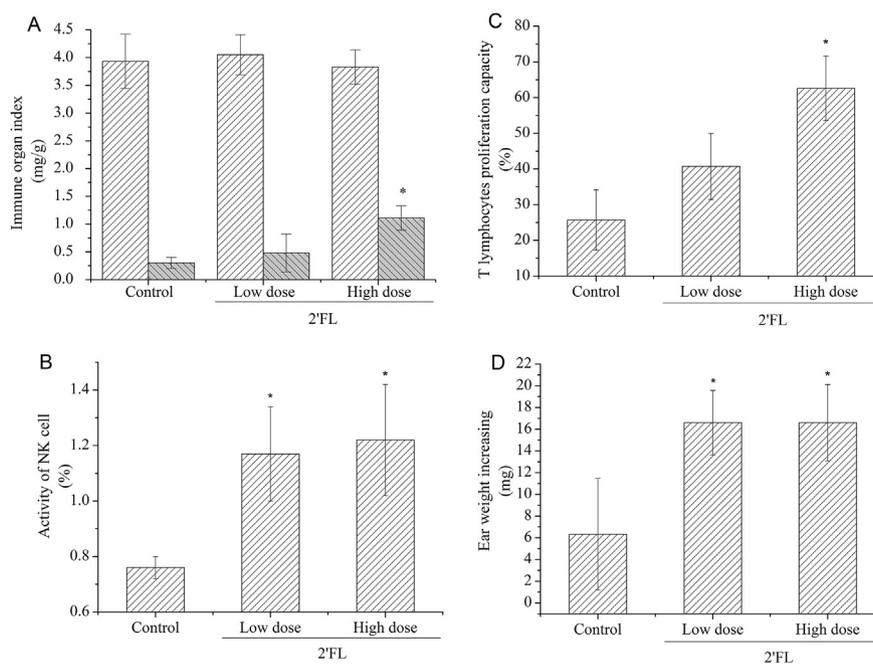


Fig. 3. The immune effect of 2'-fucosyllactose on immune organs (A:▨, spleen index; ▨, thymus index), NK cell activity (B), T lymphocytes proliferation capacity (C) and delayed-type hypersensitivity reactivity (D) in infant formula milk diet mice. The low and high groups were gavaged with 1 and 2 g kg⁻¹ of 2'-fucosyllactose once daily for 30 days, respectively. The control mice were gavaged with 10 g kg⁻¹ normal saline. Values are expressed as the mean ± SEM of 5 mice in each group of each parameter; an asterisk indicates a significant difference relative to the control group ($P \leq 0.05$).

3.5. 2'-Fucosyllactose increases the degree of delayed-type hypersensitivity reactivity in mice

DTH reactivity happens when the body re-encounters anaphylaxis; the degree of DTH reactivity is an indicator of cellular immunity function. Ear weight increase was used to reflect the degree of DTH. The degree of DTH was significantly improved in both doses of 2'FL groups compared with the control group, the average values (mg) of ear weight increase in control, low and high groups were 6.33 ± 5.15 , 16.60 ± 2.97 and 16.60 ± 3.50 , respectively ($p < 0.05$, $n = 5$ in each group; Fig. 3D).

4. Discussion

Within the study, daily supplemented 2'FL increased the thymus index, NK cell activity, T lymphocytes proliferation capacity and DTH reactivity over a period of 30 days in mice, which together implies support in immune function in mice. The immune indicators including splenic NK cell activity and the immune organ index were firstly assessed. As shown in Table 1, the body weight and the total energy were not significantly different in the three experimental groups, but there was a significant proportion of total caloric intake of 2'FL in low and high 2'FL treatment groups, compared with control (about 0%, 0.59% and 1.17%, respectively). Oku and Nakamura (2002) reported that oligosaccharide had low caloric content. The available energy of a non-digestible oligosaccharide, which is completely fermented by intestinal microbes, is estimated to be approximately 2 kcal g^{-1} (Oku & Nakamura, 2002); this might be partly the reason why the significant proportion energy of 2'FL did not affect the total caloric intake.

Vazquez, Santos-Fandila, Buck, Rueda, and Ramirez (2017) showed that 2'FL could be absorbed into the systemic circulation in rats in a dose-dependent manner. As 2'FL is minimally digested in the gastrointestinal tract, only small quantities of 2'FL were absorbed (Triantis et al., 2018). It is possible that dietary 2'FL may trigger immune function via intestine microbe or the small amount of absorbed 2'FL in a murine model (Donovan & Comstock, 2016). The thymus is the major site of T cell differentiation, development, and maturation, while the spleen plays an essential role in the synthesis of immune cells and monocytes (Li et al., 2018). Therefore, the thymus and spleen indices reflect the status of the body's immune function. The high dose of 2'FL treatment significantly increased the thymus index, while the spleen index did not show any statistically significant difference. This suggested the thymus might have a role in immune modulation triggered by the treatment. Further analysis on the effect of 2'FL in the thymus is needed.

The intestinal barrier function is considered to be the first line of defence in innate immunity (Donovan & Comstock, 2016). 2'FL was reported to increase intestinal cell maturation and increase the barrier function (Holscher, Davis, & Tappenden, 2014). However, little is known about the effect of 2'FL on two important innate immune cells, NK cells and macrophages. Due to the structural similarity of 2'FL to selectin ligand, an important mediator in immune response (Bode, 2006), potential 2'FL receptors are expressed on some immune cells (Triantis et al., 2018). It is possible that 2'FL could bind to immune cells and trigger signalling that resulted in changes to immune cell populations and functions (Donovan & Comstock, 2016). 2'FL has been demonstrated to bind to dendritic cell-specific intercellular adhesion molecule-3-grabbing-non-integrin (DC-SIGN) (Noll et al., 2016). This suggested that 2'FL may directly interact with the innate immune cells and subsequently affect adaptive immunity (Xiao et al., 2018).

NK cells are important innate immune cells that kill microorganism-infected cells. In recent years, a study showed

the amount of NK cell in peripheral blood was nearly twice in HMOs (containing 2'FL)-fed group than the formula-fed group in Neonatal Piglet model (Comstock et al., 2017). Our study provides further evidence on the effect of 2'FL on NK cells; the activity of splenic NK cell in the two groups revealed a potential strong correlation of 2'FL and NK cell, which suggests 2'FL has a good effect on natural immunity function in infant formula milk diet matrix. However, the phagocytic function of celiac macrophage (data not shown), which is also an indicator towards native natural immunity, did not show any significant difference in our study.

T cell proliferation capacity and DTH reactivity are important indicators for cellular immunity function. Comstock et al. (2014) showed that stimulation with HMOs increased lymphocyte proliferation in blood responding to mitogen such as lipopolysaccharide and phytohaemagglutinin. Here, we expanded the mitogen and used ConA to stimulate T cell proliferative response from the spleen (Yabuuchi et al., 2017); we got a similar result in mice receiving a high dose of 2'FL. Xiao et al. (2018) also reported increased T lymphocyte proliferation in an influenza mice model. This showed a strong correlation of 2'FL and the proliferation function of T cell. Our results indicated 2'FL could improve T lymphocyte proliferation without other HMOs.

DTH reactions are *in vivo* immune inflammatory responses that are due to extravascular recruitment of effector T cells (Ptak, Herzog, & Askenase, 1991). The mechanism of DTH in immune response were widely reported (Ptak et al., 1991). The DTH model sensitized by dinitrofluorobenzene in mice as well as the ear weight measurement has been proved reliable to reflect DTH reaction (Corsini et al., 1979). This model was applied to examine the immune modulatory function of M-smegmatis (Xu et al., 2005), the influence of systemic administration of neuroactive drugs on DTH (Giinther & Clansing, 1991), to partly determine the effect of 2'FL on vaccination responsiveness (Xiao et al., 2018). In our study, the DTH test was conducted *in vivo* to detect the effect of 2'FL in the matrix of infant formula milk diet on immunity. The increasing ear weight in 2'FL treatment groups showed an improvement of DTH. DTH provides protection against intracellular pathogens or other antigens in clinical (Narendra et al., 2016). Our results showed that 2'FL can enhance the host defence immune capacity against antigen (dinitrofluorobenzene) in mice. Xiao et al. (2018) reported an increased influenza-specific DTH response after feeding 2'FL; we found increasing DTH in no-disease-mice after administering 2'FL by gavage. The improvement of T cell proliferation, in conjunction with the increased DTH reactivity supports the enhancement of cellular immunity. A potential limitation of the present study was the lack of further analysis on mechanism of the interaction between 2'FL and its receptors in those immune cells.

5. Conclusions

Overall, our study expanded and added evidence on the immune effect of 2'FL. This is based on the increased thymus index, NK cell activity and cellular immunity in infant formula milk diet mice. Hence, 2'FL improves the immunity in mice that were fed with the basic infant formula milk diet. Further studies on the mechanism and clinical application of 2'FL are needed.

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