

Effect of Lacto-*N*-biose I on the Antigen-specific Immune Responses of Splenocytes

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We examined the effect of lacto-*N*-biose I (LNB) on Antigen (Ag)-specific responses of immune cells. LNB exposure *in vitro* suppressed Ag-specific Interleukin (IL)-4 secretion of mouse splenocytes significantly. However, IL-4 secretion from CD4⁺ T cells stimulated with anti-CD3 ϵ did not change significantly with LNB exposure. Additionally, Ag-specific Th1 cytokines did not change. Therefore LNB might suppress Ag-specific IL-4 through modification of Ag-presenting cells (APCs) in a manner independent of Th1-type immune development.

Key words: LNB; IL-4; naïve immune cells; anti-allergy

Common structures in biologically functional sugar chains often interact with particular molecules causing various responses. Lacto-*N*-biose I (Gal β 1-3GlcNAc, LNB) is one of such common structural units in glycoconjugates. LNB exists at the non-reducing end of the type I core structures of human milk oligosaccharides (HMOs) [1, 2]. Discovery of an LNB-specific metabolizing pathway in *bifidobacteria* has suggested that LNB behaves as a growth-promoting factor, explaining their rapid intestinal colonization in breast-fed infants [3–5]. Oral administration of LNB is expected to help the growth of *bifidobacteria* [6, 7], especially for bottle-fed infants. A practical enzymatic method has been developed to produce LNB as a food ingredient [8].

Recent studies suggested that some oligosaccharides modulate immune reactions directly [9–12]. Considering the utilization of LNB as a food material, such direct effect should be examined. The present study was conducted to investigate the immunomodulatory functions of LNB using naïve immune cells from DO11.10 mice, which express the T cell receptor (TCR) specific for Chicken ovalbumin (OVA). The DO11.10 mice were purchased from The Jackson Laboratory (Boston, MA, USA) and maintained in our specific pathogen-free animal facilities. The animal studies were reviewed and approved by the Animal Care and Use Committee of the National Food Research Institute,

National Agriculture and Food Research Organization (NARO), Japan.

The medium used for cell culture was RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 2-mercaptoethanol (50 μ M), penicillin (100 U/mL), streptomycin (100 μ g/mL) and 10% fetal calf serum (Biowest, Nuaille, France). LNB [8] was dissolved in sterile PBS at a concentration of 2 mg/mL and was stored at –30°C as a stock solution. The LNB stock solution was diluted with culture medium immediately before use.

Single-cell suspensions of splenocytes were prepared from individual DO11.10 mice by mechanical dispersion and suspended at 1×10^6 cells/mL with culture medium in 96-well culture plates (Nunc, Boston, MA, USA). Splenocytes were treated with 12.5, 25, 50 or 100 μ M of LNB, and 7.5 μ M of intact OVA (fraction V grade, Sigma-Aldrich) for up to 72 hr.

An Ag-specific cytokine secretion assay was performed in a total volume of 300 μ L per well. Culture supernatants were collected at 48 hr (for measurement of IL-2, IL-4 and IL-5) and at 72 hr (for measurement of IL-6, IL-12p40 and IFN- γ) after OVA stimulation. Supernatants were stored at –30°C until use. Cytokines were measured using Mouse Cytokine ELISA Ready-SET-Go! kits (eBioscience, San Diego, CA, USA). CD4⁺ T cells were prepared from pooled splenocytes from four DO11.10 mice by a MACS[®] system (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4⁺ T cells (1×10^6 cells/mL) separated by anti-mouse CD4 labeled MACS MicroBeads were stimulated with anti-CD3 ϵ mAb (145-2C11, eBioscience, San Diego, CA, USA)

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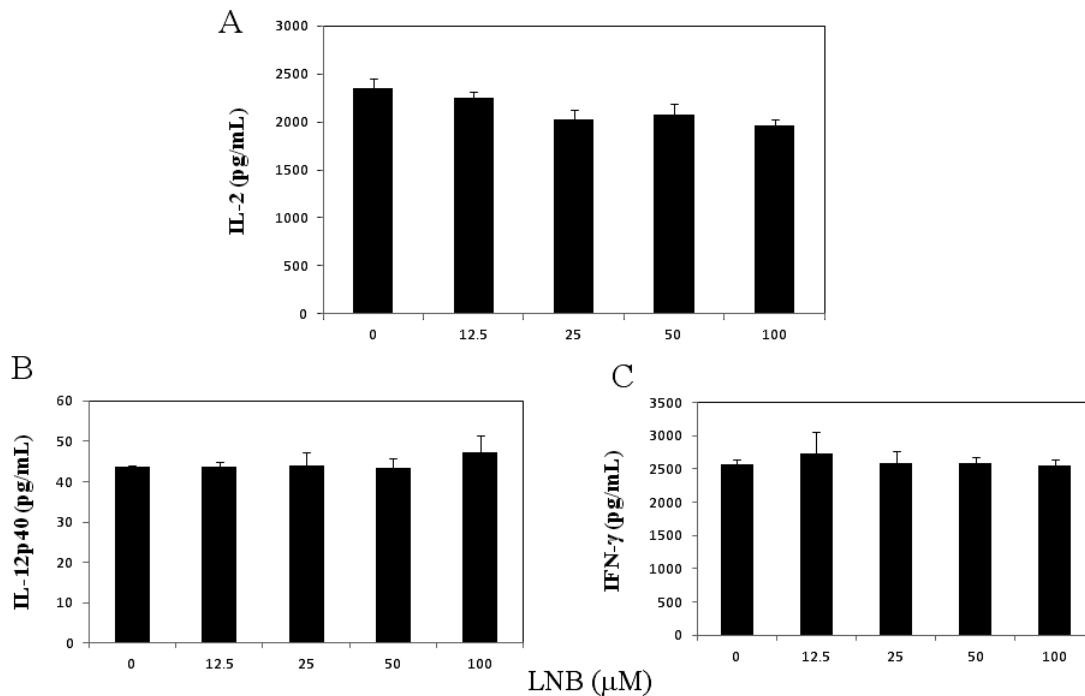


Fig. 1. The effect of LNB on antigen-specific Th1-type cytokine (A, IL-2; B, IL-12p40; C, IFN- γ) production by splenocytes from naïve mice.

Pooled splenocytes (3×10^5 cells/well) were cultured with 0 (control), 6.25, 25 or 100 μ M of LNB in the presence of 7.5 μ M of OVA in 96-well plates for 72 hr. Cytokine concentrations were measured using Mouse ELISA Ready-SET-Go! kits. The data shown are means \pm SE from triplicate cultures. (Similar results were seen in three additional experiments with three to four mice.) Statistical comparisons were carried out using ANOVA with Dunnett's multiple comparison of means test.

immobilized on plastic plate (0.5 μ g/mL of anti-CD3 ϵ in PBS was incubated for overnight at 4°C) for 48hr and 72 hr in the presence of LNB in a total volume of 300 μ L per well. Culture supernatants were collected for measurement of IL-2, IL-4, IL-5, IL-6 and IFN- γ .

The effect of LNB exposure on secretion of Ag-specific Th1-type cytokines (IL-2, IL-12p40, and IFN- γ) and Th2-type cytokines (IL-4, IL-5 and IL-6) by splenocytes is shown in Figures 1 and 2. LNB exposure reduced Ag-specific IL-4 secretion significantly (Fig. 2A). IL-4 is the most important cytokine with respect to Ag-specific Th2 cell development. On the other hand, LNB did not change IL-4 secretion from splenic T cells stimulated with anti-CD3 ϵ mAb (Fig. 3). Hence, LNB might suppress IL-4 secretion through modulation of antigen-presenting cell (APCs) functions.

LNB exposure did not change Ag-specific secretion of IL-12p40 and IFN- γ from splenocytes (Fig. 1B, C). IFN- γ is a representative Th1-type cytokine, and IL-12p40 is a subunit of bioactive IL-12 (IL-12p70), which is an essential inducer of Th1-type T cells that prevent Th2

immune cells development. We concluded that LNB exposure prevented IL-4 secretion without dependence on Th1-type cytokines.

Several oligosaccharides are recognized by pattern-recognition receptors (PRRs) including toll-like receptors (TLRs) and C-type lectin receptors [11, 12], which are expressed on APCs. Additionally, our results suggest that LNB exposure could not downregulate IL-4 secretion from T cells without signals from APCs. Therefore, LNB exposure might modify signals from APCs to T cells.

Galectins belong to lectins that have an affinity for β -galactosides and are secreted from the various cells to regulate immune cells homeostasis and inflammation [13–15]. Galectin-3 (Gal-3) is a chimera-type member of the galectin family and is associated with a proinflammatory role in fibrotic diseases [16, 17] and promotes macrophage activation [18, 19]. And some reports have indicated refreshing absence of Gal-3 leads to the development of a heightened Th1 response (IL-4 downregulation and IFN- γ upregulation) [20, 21].

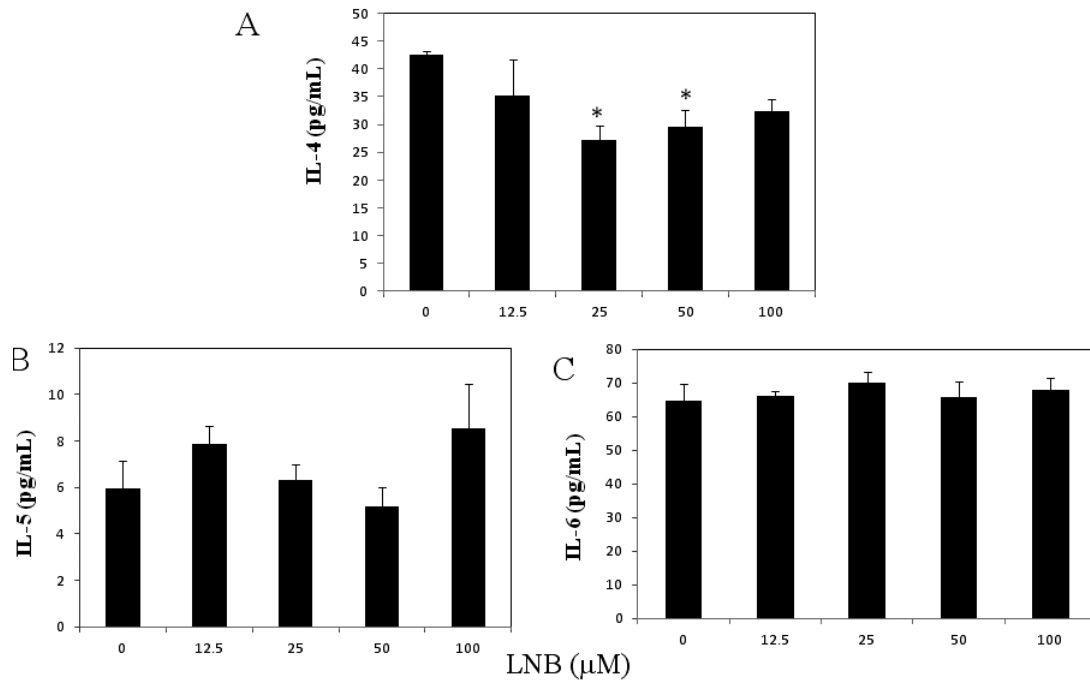


Fig. 2. The effect of LNB on antigen-specific Th2-type cytokines (A, IL-4; B, IL-5; C, IL-6) production by splenocytes from naïve mice.

Pooled splenocytes (3×10^5 cells/well) were cultured with 0 (control), 6.25, 25 or 100 μ M of LNB in the presence of 7.5 μ M of OVA in 96-well plates for 72 hr. Cytokine concentrations were measured using Mouse ELISA Ready Set Go! kits. The data shown are means \pm SE from triplicate cultures. (Similar results were seen in three additional experiments with three to four mice.) Statistical comparisons were carried out using ANOVA with Dunnett's multiple comparison of means test; comparison with the control showed significant difference (* $p < 0.05$).

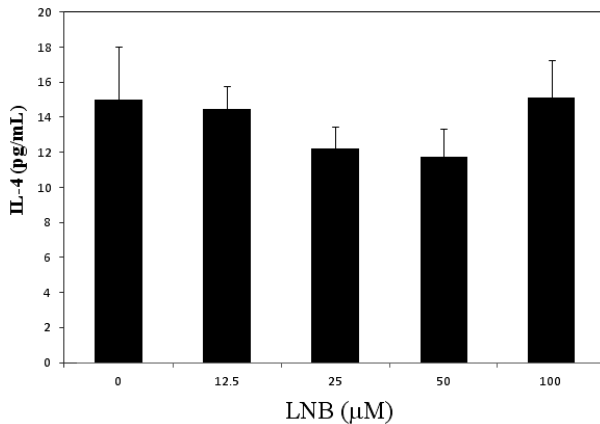


Fig. 3. The effect of LNB on IL-4 production of splenic CD4⁺ T cells stimulated with anti-CD3 ϵ antibody from naïve transgenic mice.

Splenic CD4⁺ T cells (1×10^5 cells/well) were cultured with 0 (control), 6.25, 25 or 100 μ M of LNB stimulated with immobilized anti-CD3 ϵ antibody in 96-well plates for 72 hr. IL-4 concentrations were measured using a Mouse ELISA Ready-SET-Go! kit. The data shown are means \pm SE from triplicate cultures. (Similar results were seen in three additional experiments with three to four mice.) Statistical comparisons were carried out using ANOVA with Dunnett's multiple comparison of means test.

Interestingly, exposure of lactose, which binds to Gal-3 disrupts association between TCR and Gal-3 and distorts the effects of Gal-3 [22], and it has been reported that LNB has affinity to Gal-3 similar to lactose [23]. Hence, we speculated that Gal-3 bound with LNB might cripple its regular functions partially and reduced Ag-specific IL-4 secretion only. In general, Gal-3 levels in lymphocytes are very low until induced by strong activating agents, and the expression of Gal-3 in T cells is low in comparison with other leukocytes [24]. Therefore, there might be a very small amount of a Gal-3 in the environment of purified T cell culture, and IL-4 secretion of CD4⁺ T cells stimulated with anti-CD3 ϵ might not be affected by LNB exposure (Fig. 3).

Hence, we concluded that through modification of signals from APCs, LNB affects Ag-specific immune responses. To develop LNB as a food ingredient, pharmacological effects of LNB should be taken in account as well as the prebiotic effects.

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