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Characteristic immune response in Peyer's patch cells induced by oral administration of *Bifidobacterium* components

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Abstract

We demonstrate immunomodulatory effects, especially those involving murine intestinal IgA secretion, in Peyer's patch cells following oral administration of *Bifidobacterium* immunomodulator (BIM) derived from sonicated *B. pseudocatenulatum* 7041. BALB/c mice were administered BIM orally for 7 consecutive days. The PP cells demonstrated upregulated secretion of total IgA including BIM-specific IgA following BIM administration. In observing the response of PP cells co-cultured with BIM, we found enhanced secretion of interferon-γ (IFN-γ) and interleukin (IL)-6 in the $CD4^+$ T cells. In contrast, IL-12 secretion by $Thy1.2^-$ PP cells was enhanced, but secretion of IFN-γ, IL-5, and IL-6 was not significantly affected. Furthermore, the population of $CD4^+$ $CD45RB^{high}$ T cells in PP increased following oral administration of BIM. These data suggest that $CD4^+$ T cells were affected by BIM administration. Overall, the results show that oral administration of BIM induced $CD4^+$ PP cells to change their expression of cell surface antigen and cytokine production.

Abbreviations: APCs – antigen presenting cells; BIM – *Bifidobacterium* immunomodulater; DC – dendritic cells; IFN-γ – interferon-γ; IL – interleukin; Mϕ – macrophage; PP – Peyer's patch; Th1 – type 1 helper T; Th2 – type 2 helper T

Introduction

The effects of probiotics, defined as live microbial food ingredients, are beneficial to health (Salminen et al. 1998). Some probiotic bacteria have been reported to have benefits in preventing food allergies and enhancing host immune responses. For example, live gram-positive bacteria (*e.g. Bifidobacterium* and *Lactobacillus*, etc.), and some components derived from these microorganisms, have been demonstrated to protect against infection and the effects of allergy in the host (Kalliomaki et al. 2001; Shida et al. 2002; Shu and Gill 2001; Silva et al. 2004). However, the interactions between probiotic bacteria and mucosal immune cells in the gut are not well understood.

Lee et al. reported that bifidobacteria have potent immunopotentiating activity among food microorganisms, and that *Bifidobacterium adolescentis* M101–4 (*B. pseudocatenulatum* 7041), derived from human intestinal microflora, had the strongest mitogenic activity on splenocytes and Peyer's patch cells (Lee et al. 1993). In addition, they found that this activity increased after disruption. Hosono et al. characterized the water-soluble immunoactive polysaccharides derived from this strain (Hosono

et al. 1997, 1998). Moreover, some bifidobacteria increased total IgA production in the intestine (Fukushima et al. 1998). Immunomodulation by *Bifidobacterium in vivo* is expected to be beneficial for human health. However, the molecular and cell biology of the immunoregulatory responses induced in the gut-associated lymphoid tissue by oral administration of *Bifidobacterium* components has not been elucidated.

The unique characteristics of the intestinal immune response are secretory IgA production and oral tolerance. Peyer's patches (PP) which are representative of lymphoid follicles in the intestinal mucosa, are the inductive sites of immune reaction to oral antigens. However, the mechanisms of immunomodulation by probiotic bacteria have not been clarified in detail. In particular, there is no data available as to how antigen-presenting cells (APCs) or $CD4^+$ T cells respond to probiotic bacteria in the PP.

In this study, we have investigated the immune responses of PP cells by oral feeding of *Bifidobacterium* immunomodulators (BIM) derived from sonicated *B. pseudocatenulatum* 7041 on mucosal immune responses in the intestine. We found that oral administration of BIM enhanced IgA production by PP cells and observed characteristic responses of $CD4^+$ T cells in PP following oral administration of BIM.

Materials and methods

Animals

Female 6–9 weeks old BALB/c mice were obtained from Clea Japan (Tokyo, Japan) and were housed in a room with a 12 h light-dark cycle. The mice were naturalized and given MF diet (Oriental Yeast, Tokyo, Japan) for 3 days before experiments. All mice were kept in accordance with Guideline for the Care and Use of Laboratory Animals by College of Bioresource Sciences, Nihon University.

Preparation of Bifidobacterium immunomodulator (BIM) derived from sonicated B. pseudocatenulatum 7041, and oral administration of BIM

Sonicated *B. pseudocatenulatum* 7041 was prepared by the method described in previous reports (Lee et al. 1993; Hosono et al. 2003). Mice were orally administered a dose of 10 mg/day of BIM in saline by using a feeding-tube for 7 consecutive days. The control mice were given saline by the same method of tube feeding. Mice were allowed free access to a pelleted MF diet and sterile deionized water throughout the experimental period.

Preparation of $CD4^+$ T cells and Thy1.2$^-$ cells from PP, and APC from splenocytes

After 7 days oral administration of BIM, PP cells were obtained from each experimental group by the method described previously (Hosono et al. 2003), and $CD4^+$ T cells were isolated by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-mouse CD4-conjugated magnetic microbeads and an LS column following the manufacturer's instructions. Thy1.2$^-$ (CD90) negative PP cells from the experimental mice were isolated by MACS with anti-mouse CD90-conjugated magnetic microbeads and LS column. APC were derived from splenocytes of BALB/c mice, which had not been given the experimental diet. The cell suspensions of splenocytes were treated with 50 μg/ml mitomycin C (Sigma, St. Louis, MO) for preparation of APC.

Flow cytometric analysis

Flow cytometric analysis of $CD4^+$ cells was performed using a FACScalibur with CellQuest software (BD Biosciences, Mountain View, CA) and staining with anti-TCR$\alpha\beta$-biotin, anti-CD4-FITC, and either PE-, anti-CD45RB, anti-CD44, or anti-CD69. Fc receptors (FcγRIII/II) were blocked using anti-mouse CD16/CD32. The above Abs were purchased from BD PharMingen (San Diego, CA) Isotype-matched negative controls were included in the analysis of the cells suspensions. Purity of $CD4^+$ cells from PP was >93% (data not shown). We checked the expression of CD90 (<0.8%) of Thy1.2$^-$ PP cells by FACS (data not shown).

Measurement of total IgA

Total IgA in the intestinal ingredients and culture supernatant was measured by sandwich ELISA.

The intestinal tracts from duodenum to rectum were dissected from the experimental mice. The intestinal tracts were homogenized with 2 ml PBS containing 50 mM EDTA and 0.1 mg/ml trypsin inhibitor, and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatants obtained from the intestinal ingredients were diluted appropriately for ELISA. PP cells from the experimental groups were plated onto the wells of a 48-well plate at 2.5×10^6 cells per well, and co-cultured for 5–7 days with 0, 10, or 50 μg/ml BIM in RPMI 1640 medium containing 5% fetal bovine serum. The amounts of total IgA in the intestinal ingredients or culture supernatants were measured by means of a sandwich ELISA method described previously (Hosono et al. 2003).

Measurement of BIM-specific IgA

BIM-specific IgA was measured by ELISA. Plates were coated with 50 μl of 100 μg/ml BIM. Subsequent handling was followed as described above. BIM-specific IgA was qualitatively determined by absorbance at 405 nm.

Culture and cytokine determinants

$CD4^+$ T cells from PP cells from experimental mice (2×10^6 cells/well) in a total volume of 1 ml were cultured with 0, 10, or 50 μg/ml BIM in the presence of APC derived from splenocytes (8×10^6 cells/well) in RPMI 1640 medium containing 5% FCS in a 48-well plate. $Thy1.2^-$ PP cells (6×10^6 cells/well) in a total volume of 1 ml were cultured with BIM. The culture supernatants were collected after 24 or 72 h and assayed for IL-12 p40 and for IL-5, IL-6, and IFN-γ respectively. The amounts of IL-5, IL-6, and IFN-γ in the supernatants were measured by means of a sandwich ELISA method described previously. IL-12 was measured using an Opt-EIA mouse IL-12 (p40) set (BD PharMingen).

Statistical analysis

Data are expressed as means ± SD. Differences were examined by one-way analysis of variance (ANOVA), and significant differences found between groups were further evaluated by Tukey's test (SPSS Ver. 10.0, Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

Results

Total IgA production and BIM-specific IgA of PP cells after BIM administration

There was no significant increase of body weight gain among the experimental groups (data not shown). The total number of PP and PP cells were not different among the experimental groups. We examined total IgA production by PP cells derived from the experimental mice during a 7-day primary cells culture with different doses of BIM. PP cells had been prepared from the experimental mice. The total IgA production by PP cells of BIM-fed groups was higher than that of control groups for all doses of BIM *in vitro* (Figure 1a). In addition, BIM-specific IgA production by PP cells was enhanced by oral administration of BIM (Figure 1b).

Cytokine production patterns of $CD4^+$ from PP cells

We examined the cytokine production patterns of $CD4^+$ T cells derived from PP cells, which were obtained from both the control mice and mice administered BIM. The prepared $CD4^+$ T cells were cultured with BIM in the presence of APC from naïve mice splenocytes *in vitro*, and the amounts of cytokine in the supernatants were measured (Figure 2). Secretion of both IFN-γ and IL-6 was enhanced by orally administered BIM. However, the secretion of IL-4 and IL-5 was not detected (data not shown).

Increased $CD4^+CD45RB^{high}$ PP T cells following orally administered BIM

To examine the effects of BIM feeding on the state of $CD4^+$ PP T cells derived from experimental mice, we analyzed surface cell antigens of purified $CD4^+$ PP cells by flow cytometic analysis. All results were gated TCR$\alpha\beta$. As shown in Table 1, the BIM-fed group shows higher expression of

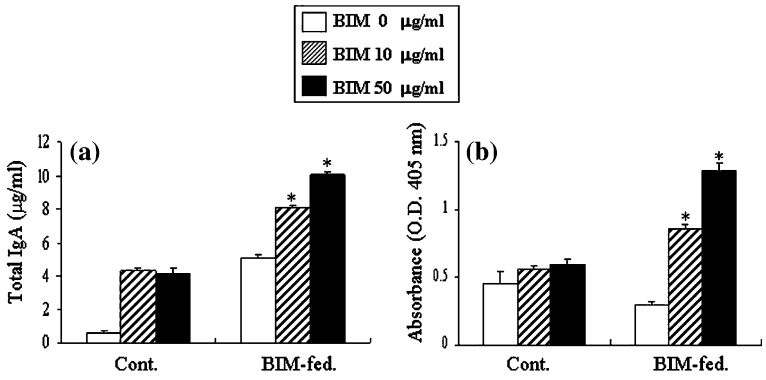

Figure 1. Effects of oral administration of BIM on total IgA production from PP cells. PP cells were obtained and pooled for each experimental group respectively, and then the cells were cultured with 0–50 μg/ml of BIM for 7 days. (a) Total IgA in the culture supernatants was measured by ELISA. (b) BIM-specific IgA in the culture supernatants was qualitatively measured by ELISA. The values are expressed as the mean of triplicate cultures ± S.D. Additional concentrations in *in vitro* culture were BIM 0 μg/ml (white bar), BIM 10 μg/ml (shadow bar), and 50 μg/ml (black bar). *Significant difference from control group of the same BIM dose at $p < 0.05$ by Tukey's test. The results shown are representative of two independent experiments.

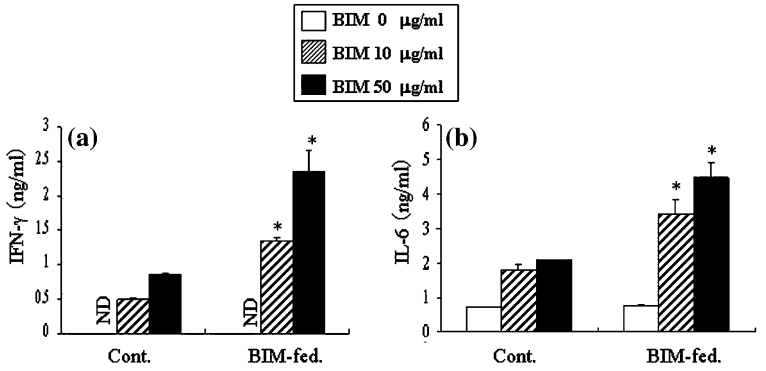

Figure 2. Effects of oral administration of BIM on cytokine production by murine PP CD4$^+$ T cells. PP CD4$^+$ T cells were obtained and pooled for each experimental group respectively. The cells were then co-cultured with 0–50 μg/ml BIM in the presence of APC for 72 h. The cytokines in the culture supernatants were measured by ELISA. The values are expressed as the mean of triplicate cultures ± S.D. ND, not detected. Additional concentrations in *in vitro* culture were BIM 0 μg/ml (white bar), BIM 10 μg/ml (shadow bar), and 50 μg/ml (black bar). *Significant difference from control group of the same BIM dose at $p < 0.05$ by Tukey's test. The results shown are representative of two independent experiments.

CD4$^+$CD45RB^{high} than control. In contrast, activation-markers CD44 or CD69 were not significantly different from control.

Cytokine production patterns of Thy1.2$^-$ cells from PP

To examine the effects of BIM administration on responses of APCs in the PP, we measured the cytokine production patterns of Thy1.2$^-$ cells prepared by negative sorting of Thy1.2$^+$ cells from PP cells obtained from experimental groups. We observed the level of IL-12 p40 on 24 h-cell cultures from orally administered BIM groups was higher than that of the control group. The secretion of IL-5, IL-6, and IFN-γ was maintained at high levels, but there were no significant differences among these groups (Figure 3).

Oral administration of BIM did not induce total IgA in the intestinal mucosa in vivo

Although we measured the total IgA secretion in the intestinal contents from the ileum to the

Table 1. Effects of oral administration of BIM on the expression of cell surface antigen on $CD4^+$ T cells.

	Cont.	BIM-fed.	*p* value
$CD4^+CD45RB^{high}$	49.6 ± 2.4	57.9 ± 6.1	0.084
$CD4^+CD45RB^{low}$	47.3 ± 2.7	39.0 ± 5.7	0.094
$CD4^+CD44^{high}$	59.5 ± 9.7	58.9 ± 8.2	0.933
$CD4^+CD44^{low}$	37.8 ± 9.3	38.2 ± 7.3	0.949
$CD4^+CD69^{high}$	40.8 ± 4.8	40.7 ± 8.4	0.975
$CD4^+CD69^{low}$	56.5 ± 3.7	55.9 ± 8.3	0.885

Purified $CD4^+$ PP cells derived from experimental mice and analyzed by flow cytometic analysis. All results were gated TCR$\alpha\beta$. Values are means of three independent experiments ± S.D and expressed as percentage of viable cells.

rectum *in vivo*, no significant increase was observed in these experimental groups (Figure 4).

Discussion

There are some reports showing that probiotic bacteria supplementation induces immunological responses in treated animals. However, it was not clear exactly how probiotic bacteria and their components modulated mucosal immune response in the gut. In particular, it has not been clearly elucidated that how helper T cells and APCs interact to IgA production respectively by oral administration of probiotic bacteria.

Probiotic bacteria enhance humoral immune responses by increasing the number of IgA producing cells (Kaila et al. 1992; Park et al. 2002) and increasing the systemic and mucosal IgA response to dietary antigen (Kaila et al. 1992; Shroff et al. 1995; Takahashi et al. 1998) In addition, *Lactobacillus*-specific IgA in PP cells cultured from conventional mice was significantly increased when compared with that in germfree mice (Ibnou-Zekri et al. 2003). We examined whether sonicated *Bifidobacterium* components that have strong mitogenic activity influenced the ability of PP cells to produce IgA. We showed that BIM administration up-regulated the proliferation activity of PP cells *in vitro* (data not shown). These results indicate that orally administered BIM was taken up by M cells on the PP and then activated PP cells. We demonstrated that BIM-feeding enhanced the production of cytokines, such as IFN-γ, IL-5, IL-6, and IL-12, in the PP cells (data not shown). We also observed that both total IgA and BIM-specific IgA production in the PP derived from BIM-fed animals was higher than that of the control group (Figure 1). Recent studies have shown that a novel cell subset in the PP secreted IL-5 and increased IgA secretion by B cells (Kuraoka et al. 2004). Previous studies have shown that the induction of $CD4^+$ cells in PP is important in the activation of mucosal IgA responses (Kiyono et al. 1992; McGhee, et al. 1992). Our results show that secretion of both IFN-γ and IL-6 by $CD4^+$ T cells were increased following BIM administration (Figure 2). On the other hand, IFN-γ, IL-5, and IL-6 secretion by $Thy1.2^-$ PP cells from experimental groups showed no significant alteration in response to BIM-feeding (Figure 3). It has been shown that orally administered antigens increase Th2 type cytokine responses in PP (Yoshida et al. 2002), and that IL-5, IL-6, or IL-10 induces the differentiation of B cells to surface IgA^+ B cells (Defrance et al. 1992; Xu-Amano et al. 1992). It has also been reported that the oral administration of lactic-acid bacteria induces IFN-γ production in mucosal lymphocytes (Aattour et al. 2002), and that IFN-γ can promote the uptake of antigens in PP, where specific IgA-related cells are generated (Sutas et al. 1997). Our results suggest that BIM-feeding could induce cytokine production in PP cells including $CD4^+$ T cells, which enhanced both total IgA and BIM-specific IgA production in the PP.

The level of expression of CD45RB on mouse $CD4^+$ T cells has been shown to distinguish naïve ($CD45RB^{high}$) from activated/memory ($CD45RB^{low}$) cells (Powrie 1995). It was reported that the cytokine profiles of $CD45RB^{high}$ and $CD45RB^{low}$ cells differ (Ten Hove et al. 2004), and that $CD45RB^{high}$ cells produce more IFN-γ (Horgan et al. 1994). We observed that $CD4^+$ PP T cells showed enhanced IFN-γ and IL-6 secretion (Figure 2), but increased naïve marker, $CD45RB^{high}$ expression (Table 1) following oral administration of BIM. Although we have not examined the responses of $CD4^+$T cells in the lamina propria as an effector site, we suspect

that $CD4^+CD45RB^{high}$ cells regulated total IgA production *in vivo*. When we examined mucosal total IgA production in the intestine *in vivo*, total IgA was not increased following oral administration of BIM (Figure 4). Further studies will be needed to elucidate the reason why the numbers of these naïve cells in the PP increased following feeding with BIM.

Although secretion of IFN-γ, IL-5 and IL-6 by $Thy1.2^-$ PP cells from each experimental group was not significantly different, it should be noted that secretion of IL-12 by these cells was enhanced by orally administered BIM (Figure 3). When $CD4^+$ T cells only were cultured with BIM, no proliferative activity was observed (data not shown). In other words, alteration of $CD4^+$ T cells following oral administration of BIM may be mediated by APCs in the PP. Because secretion of IL-12 was enhanced by BIM feeding (Figure 3), this naturally implies that dendritic cell (DC) or

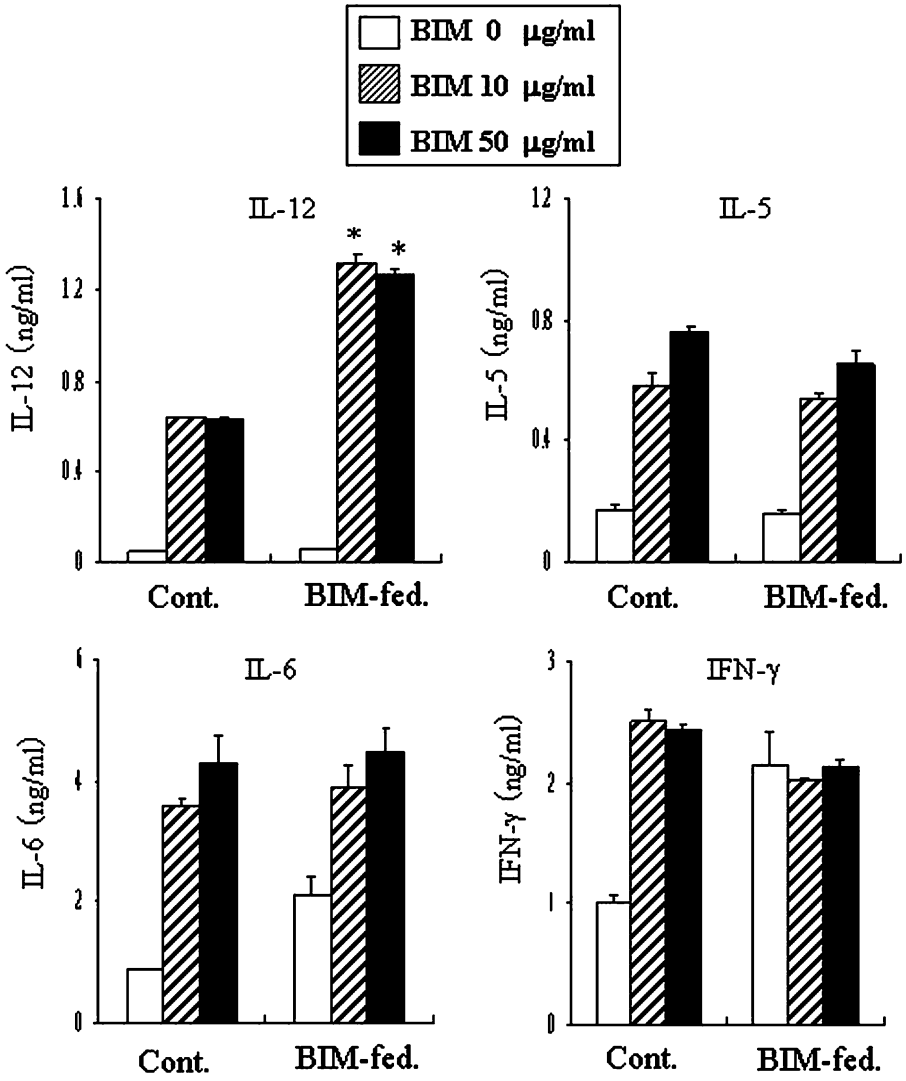

Figure 3. Effects of orally administered BIM on cytokine production from murine $Thy1.2^-$ PP cells. $Thy1.2^-$PP cells were obtained and pooled for each experimental group respectively. The cells were then cultured with 0–50 μg/ml of BIM for 24 or 72 h. The cytokines in the culture supernatants were measured by ELISA. The values are expressed as the mean of triplicate cultures ± S.D. Additional concentrations in *in vitro* culture was BIM 0 μg/ml (white bar), BIM 10 μg/ml (shadow bar), and 50 μg/ml (black bar). *Significant difference from control group of the same BIM dose at $p < 0.05$ by Tukey's test. The results shown are representative of two independent experiments.

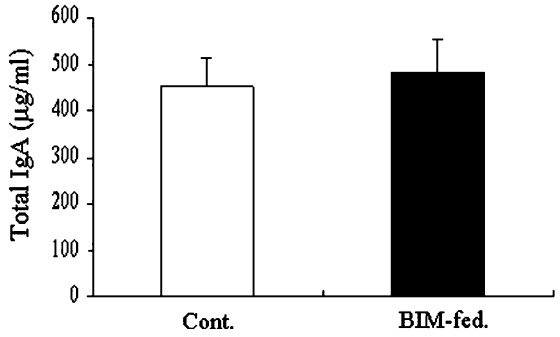

Figure 4. Effects of orally administered BIM on total IgA production in the intestine *in vivo*. After 7 days of oral administration of BIM, total IgA levels in the intestinal tract were measured by ELISA. The intestinal tracts were obtained from experimental group. The values are expressed as the means ± S.D. ($n = 9$). The results shown are representative of three independent experiments.

macrophage (Mϕ) in the Thy1.2$^-$ PP cells was activated by BIM in the initial phase. Cells of the innate immune system, such as DC and Mϕ, use a variety of pathogen-associated molecular pattern (PAMP) recognition receptors to recognize the patterns shared between pathogens (Janeway 1998; Banchereau et al. 2000). PAMPs include lipopolysaccharide from Gram-negative bacteria, lipoteichoic acid, lipoarabunomannan, lipoprotein, and unmethylated DNA with a CpG motif. These cause Mϕ and DC to produce a set of proinflammatory cytokines, such as tumor necrosis factor α, IL-1, IL-6 and IL-12. They also induce the maturation of DC which driveTh1 cell development leading to the activation of adaptive immunity (Takeuchi and Akira 2002). Recently, Toll-like receptors (TLR) were identified as major recognition receptors for PAMP (Poltorak et al. 1998; Yang et al. 1998; Hemmi et al. 2000). In particular, TLR2 responds to Gram-positive cell wall components (Yoshimura et al. 1999). In the intestinal mucosa, we speculate that DC in the PP recognize BIM, including peptidoglycan from Gram-positive bacteria, via TLR2, and then activate the adaptive immune response.

The PP has been considered to be the major inductive site for the initiation of high affinity secretory IgA immune responses in the gastrointestinal tract. In this study, we showed that orally administered BIM enhanced total IgA production including BIM-specific IgA from PP cells. We believe that BIM can modulate the cellular immune responses in PP via the adjuvant-like activity of BIM. Some live *Bifidobacterium* as well as this component derived from them have adjuvant activity (Sekine et al. 1994). Meanwhile, the level of total IgA in the intestinal contents did not increase significantly following BIM feeding *in vivo*. This suggests that BIM might not be excluded from the host because BIM was derived from a non-pathogenic bacterium, which was permitted symbiosis. Although, oral feeding of BIM did not induce higher levels of total IgA production in conventional conditions *in vivo*, we speculate that BIM will induce protective immune responses if pathogenic bacteria invade the gut.

In this study, we demonstrated that BIM, acting as a probiotic immunomodulator, induced mucosal IgA secretion in PP, and induced CD4$^+$ PP T cells to change the expression of cell surface antigens and cytokine secretion. In addition, since BIM is not pathogenic and is derived from a bacterium symbiotic with human intestinal commensal bacteria, IgA hyperproduction in the mucosa might be regulated in the healthy host, although the heightened ability for IgA production is maintained in the PP cells. It will be interesting to clarify the role of probiotic bacteria in the modulation of the adaptive immune response via the innate immune response. Further study will be needed to clarify this clear interaction between probiotic bacteria and immune cells.

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