

RESEARCH ARTICLE

Suppressive effects of *Bifidobacterium longum* on the production of Th2-attracting chemokines induced with T cell–antigen-presenting cell interactions

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Abstract

In human trials, *Bifidobacterium longum* BB536 alleviates subjective symptoms of Japanese cedar pollinosis, an IgE-mediated type I allergy caused by exposure to Japanese cedar, and significantly suppresses the increase of plasma thymus- and activation-regulated chemokine (TARC) associated with pollen dispersion. In the present study, we investigated the suppressive effects of BB536 on the production of T helper type 2 (Th2)-attracting chemokines, such as TARC and macrophage-derived chemokine (MDC), together with the mechanisms of their production. Murine splenocytes were cultured with heat-killed BB536, and the levels of Th2-attracting chemokines in the supernatants were measured. TARC and MDC were produced in cultures without stimulation, and the production was significantly suppressed by BB536. These chemokines were produced by antigen-presenting cells (APCs) of splenocytes stimulated with an anti-CD40 antibody. Furthermore, TARC production was induced with granulocyte macrophage colony-stimulating factor that was produced by T cells and dendritic cells. BB536 suppressed MDC production induced with the anti-CD40 antibody by APCs from the spleen, mesenteric lymph nodes (MLNs) and Peyer's patches, and it suppressed TARC production by APCs from the spleen and MLNs. These results indicate that BB536 suppresses the production of Th2-attracting chemokines induced by the T cell–APC interaction, suggesting a novel mechanism for alleviating symptoms of allergic disorders by probiotics.

Introduction

Probiotics are live bacteria that have beneficial effects on human health (FAO/WHO, 2002). There is substantial interest in the antiallergic activity of probiotics. *Bifidobacterium longum* BB536 was originally isolated from a human fecal sample, and has been used in the dairy industry as a probiotic bacterium. In human trials, BB536 alleviates subjective symptoms of Japanese cedar pollinosis, an IgE-mediated type I allergy caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen (Xiao *et al.*, 2006a,b, 2007b), and significantly suppresses the increase of plasma thymus- and activation-regulated chemokine (TARC, CCL17) associated with pollen dispersion (Xiao *et al.*, 2006b, 2007a).

T helper type 2 (Th2)-attracting chemokines, such as TARC and macrophage-derived chemokine (MDC, CCL22), selectively attract Th2 memory T cells via the CC chemokine receptor 4 (CCR4) (Imai *et al.*, 1999). Plasma TARC levels in patients with allergic diseases, such as atopic dermatitis (AD), bronchial asthma and allergic rhinitis, are significantly higher than those of healthy subjects (Sugawara *et al.*, 2002). Moreover, plasma TARC and MDC levels were significantly correlated with severity scores for AD (Fujisawa *et al.*, 2002; Horikawa *et al.*, 2002), and the administration of anti-TARC antibodies diminished the degree of airway hyperresponsiveness in a murine model of allergic asthma (Kawasaki *et al.*, 2001). These reports suggest that regulating the production of Th2-attracting chemokines is important for alleviating the symptoms of allergic disorders.

Allergic disorders are characterized by an immediate hypersensitivity reaction, followed by a late inflammatory reaction (late-phase response). The late inflammatory reaction, which occurs several hours after contact with the allergen, is characterized by tissue infiltration by antigen-presenting cells (APCs), eosinophils and memory T lymphocytes (mainly Th2 cells) that contribute to tissue damage (Kaplan, 2001; Allam *et al.*, 2005). This infiltration in the late-phase response is tightly controlled by Th2-attracting chemokines, such as TARC and MDC. These chemokines are produced in inflammatory regions by dendritic cells (DCs) and epidermal keratinocytes (Saeki & Tamaki, 2006), and histamine, which is released from IgE-activated mast cells, acts on DCs to produce Th2-attracting chemokines (McIlroy *et al.*, 2006). Locally recruited Th2 cells produce Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, which activate eosinophil functions, including migration (Lampinen *et al.*, 2004; Kagami *et al.*, 2005). Locally recruited eosinophils produce chemoattractants for themselves, such as RANTES and eotaxin, and release toxic granule proteins and oxygen free radicals (Lampinen *et al.*, 2004). Furthermore, in this serial stream of an allergic reaction, Th2 cytokines, such as IL-4 and IL-13, produced by locally recruited Th2 cells also promote the production of Th2-attracting chemokines by DCs (Fujita *et al.*, 2005). Thus, allergic reactions are complex processes caused by interactions between many cells and mediators of the immune system, and the production of Th2-attracting chemokines plays a crucial role in these allergic reactions. However, the mechanism by which Th2-attracting chemokines are produced in interactions between immunocompetent cells has not been completely elucidated.

In the present study, we investigated the suppressive effects of BB536 on the production of TARC and MDC, together with the mechanisms of their production. Our results suggest that the T cell–APC interaction induced the production of TARC and MDC by APCs and that heat-killed BB536 suppressed the production of these chemokines.

Materials and methods

Mice

Male BALB/c mice were purchased from Charles River Japan (Yokohama, Japan) and were used for experiments at 7–10 weeks of age. Mice were maintained in the animal facility at the University of Tokyo and were used in accordance with the guidelines of the University of Tokyo.

Bacteria

Bifidobacterium longum BB536 was obtained from the collection held at Morinaga Milk Industry Co. Ltd (Zama, Japan). Unless otherwise noted, BB536 was cultured for 16 h

at 37 °C in lactobacilli-MRS broth (Difco, Detroit, MI), collected by centrifugation, washed twice with phosphate-buffered saline (PBS) and then washed twice with sterile distilled water. The microorganisms were lyophilized and suspended in PBS and were then killed by heating at 100 °C for 30 min. One microgram of lyophilized BB536 contained 9.8×10^5 microorganisms.

In an experiment comparing different bacteria, *Enterococcus faecalis* JCM 5803^T, *Escherichia coli* JCM 1649^T and *Bacteroides fragilis* JCM 11019^T (all were from the Japan Collection of Microorganism, Wako, Japan) were used. These bacteria were cultured in GAM broth (Nissui, Tokyo, Japan) containing 1% glucose, and BB536 was cultured in lactobacilli-MRS broth. After incubation for 16 h at 37 °C, the cultures were killed by heating at 60 °C for 30 min, collected by centrifugation, washed twice with PBS and then suspended in PBS. One microgram of lyophilized *E. faecalis*, *E. coli* and *B. fragilis* contained 1.9×10^6 , 1.4×10^6 and 6.2×10^5 microorganisms, respectively.

Antibodies and reagents

We used the following antibodies: American hamster anti-mouse CD3ε monoclonal antibody (mAb) 145-2C11 (BD PharMingen, San Diego, CA), Syrian hamster anti-mouse CD28 mAb 37.51 (BD PharMingen), rat anti-mouse CD40 mAb 1C10 (R&D systems, Minneapolis, MN), rat anti-mouse granulocyte macrophage colony-stimulating factor (GM-CSF) mAb MP122E9 (R&D systems), rat anti-mouse interferon (IFN)-γ mAb 37895.11 (R&D systems), rat anti-mouse IL-1β mAb 30311.11 (R&D systems), rat anti-mouse IL-4 mAb 30340.11 (R&D systems), rat anti-mouse IL-6 mAb MP520F3 (R&D systems), rat anti-mouse IL-10 mAb JES052A5 (R&D systems), rat anti-mouse IL-12 mAb C17.8 (Biolegend, San Diego, CA), rat anti-mouse IL-18 mAb 93-10C (Medical & Biological Laboratories, Woburn, MA), rat anti-tumor necrosis factor-α (anti-TNF-α) mAb MP6-XT22 (Biolegend), rat IgG1 isotype control mAb 43414.11 (R&D systems) and rat IgG2a isotype control mAb 54447.11 (R&D systems). Recombinant mouse (rm) GM-CSF and rmTNF-α were purchased from R&D systems.

Cell preparation and cell culture

To obtain ovalbumin-sensitized splenocytes, male BALB/c mice were injected intraperitoneally with 50 μg of ovalbumin (grade V; Sigma, St. Louis, MO) and 1 mg of Al(OH)₃ (Wako Pure Chemical, Tokyo, Japan); splenic cells were prepared after 10 days.

Single-cell suspensions of splenocytes and mesenteric lymph node (MLN) lymphocytes were prepared by pressing spleen tissue and MLNs through a cell strainer (BD, Franklin Lakes, NJ) with a plunger from a 5-mL polypropylene

syringe. Peyer's patches (PPs) were digested with collagenase type I (Sigma), and single-cell suspensions were prepared. For preparation of splenic DCs, spleen tissue was digested with collagenase to obtain single-cell suspensions. Erythrocytes in splenocytes were depleted with Tris-buffered NH_4Cl solution.

T cells (CD90.2^+ cells), CD90.2^- cells, major histocompatibility complex (MHC) class II $^+$ cells, B cells (B220^+ cells), macrophages (CD11b^+ cells) and DCs (CD11c^+ cells) were isolated using the MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocols. The purity of these fractions as determined by flow cytometry was $> 90\%$, $> 90\%$, $> 88\%$, $> 95\%$, $> 90\%$, and $> 85\%$, respectively.

Cells were prepared at a dilution of 2.5×10^6 cells mL^{-1} in RPMI 1640 containing 10% fetal calf serum, 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, 50 μM 2-mercaptoethanol and 0.03% L-glutamine. Unless otherwise noted, cells were cultured with heat-killed bacteria and reagent for 7 days at 37°C in 5% CO_2 . The culture supernatants were collected and kept frozen until use. No cytotoxic effect of the heat-killed bacterial cells was found on the cell viability of splenocytes as examined according to trypan blue exclusion (data not shown).

Anti-CD40 antibody ($5 \mu\text{g mL}^{-1}$) was added to stimulate CD90.2 -cells and APCs. T cells were stimulated with plate-coated anti-CD3 antibody ($5 \mu\text{g mL}^{-1}$) and with the addition of anti-CD28 antibody ($10 \mu\text{g mL}^{-1}$). An isotype-matched control antibody for the anti-CD40 antibody was used in control experiments. In the neutralization study, $5 \mu\text{g mL}^{-1}$ of neutralizing antibody or isotype-matched control antibody was added.

Measurement of cytokines and chemokines

Cytokines (GM-CSF, IFN- γ , IL-12p40 and IL-12p70) and chemokines (TARC and MDC) in the culture supernatants

were quantified using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems) in accordance with the manufacturer's instructions.

Statistics

Data are expressed as mean $\pm$ SD. Statistical analyses were performed with Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

Results

BB536 suppresses the production of Th2-attracting chemokines by splenocytes

To examine whether TARC and MDC were produced by murine splenocytes in an antigen-specific reaction and, if so, whether BB536 suppressed this production, splenocytes from ovalbumin-sensitized mice were cultured with or without heat-killed BB536 in the absence or presence of ovalbumin (Fig. 1a). Although the levels of TARC and MDC produced in the presence of ovalbumin were higher than those in the absence of ovalbumin, these chemokines were produced by splenocytes without antigen stimulation. BB536 clearly suppressed these productions in the presence of ovalbumin.

Because the production of TARC and MDC was induced in a non-antigen-specific reaction, splenocytes from mice without immunization were used to elucidate the mechanisms of this production in further experiments. Splenocytes from mice without immunization were cultured with or without heat-killed BB536 (Fig. 1b and c). TARC and MDC were produced by splenocytes without antigen stimulation, and BB536 suppressed the production of these chemokines in a dose-dependent manner.

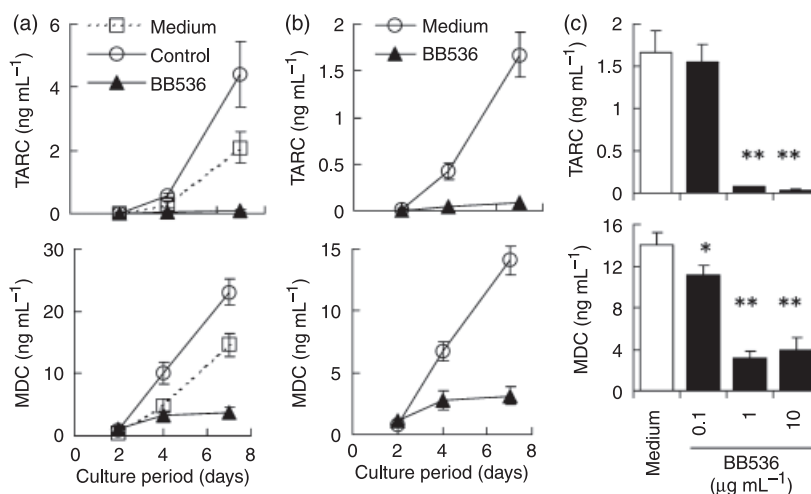


Fig. 1. BB536 suppresses the production of Th2-attracting chemokines by splenocytes. (a) Splenocytes from ovalbumin-sensitized mice were cultured with or without BB536 ($1 \mu\text{g mL}^{-1}$) in the absence (medium) or presence (control) of ovalbumin ($100 \mu\text{g mL}^{-1}$). (b, c) Splenocytes without ovalbumin sensitization were cultured with or without (medium) $1 \mu\text{g mL}^{-1}$ of BB536 (b) or 0.1–10 $\mu\text{g mL}^{-1}$ of BB536 (c). The levels of Th2 chemokines in the supernatants after indicated periods (a, b) or on day 7 (c) were measured using ELISA. Data are shown as mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with control.

Interactions with T cells induce the production of Th2-attracting chemokines by APCs

To elucidate the mechanism of the production of Th2-attracting chemokines by unstimulated splenocytes, we examined the production of TARC and MDC by splenocytes separated by CD90.2 expression. CD90.2⁺ cells are considered to be T cells, and CD90.2⁻ cells are mainly APCs. CD90.2⁺ cells alone and CD90.2⁻ cells alone did not produce TARC or MDC; these chemokines were produced in a mixture of CD90.2⁺ cells and CD90.2⁻ cells (Fig. 2a). CD90.2⁺ cells stimulated with plate-coated anti-CD3 ϵ antibodies and anti-CD28 antibodies did not produce TARC and MDC, but CD90.2⁻ cells stimulated with anti-CD40 antibodies clearly produced these chemokines (Fig. 2b).

Because CD90.2⁻ cells include minor cell populations that are not APCs, such as natural killer cells, the production of Th2-attracting chemokines by APCs that expressed MHC class II⁺ was compared with that by CD90.2⁻ cells. MHC class II⁺ cells, as well as CD90.2⁻ cells, produced TARC and MDC when stimulated with anti-CD40 antibodies (Fig. 2b). These results indicate that interaction with T cells, especially CD40-mediated signals, induces the production of Th2-attracting chemokines by APCs.

BB536 suppresses the production of Th2-attracting chemokines by APCs from spleen, MLNs and PPs

BB536 significantly suppressed the production of TARC and MDC by CD90.2⁻ cells from the spleen and MLNs that were stimulated with anti-CD40 antibodies (Fig. 3a and b).

Although at a reduced level in comparison with the spleen and MLNs, BB536 suppressed MDC production and tended to suppress TARC production by CD90.2⁻ cells from PPs (Fig. 3c). BB536 suppressed the production of TARC and MDC by splenic MHC class II⁺ cells that were stimulated with anti-CD40 antibodies; this suppression was similar to the suppression in CD90.2⁻ cells from the spleen (data not shown).

Effects of other bacteria on the production of Th2-attracting chemokines by CD90.2⁻ cells

To compare the effect of different bacteria on the production of Th2-attracting chemokines, CD90.2⁻ splenocytes were cultured with BB536, *E. faecalis*, *E. coli* and *B. fragilis* that were killed by heating at 60 °C (10 μ g mL⁻¹) in the presence of anti-CD40 antibody (Fig. 4). The levels of TARC production were decreased by the addition of all the strains. The levels of MDC production were decreased by the addition of BB536 and *E. faecalis*, but were increased by the addition of *E. coli* and *B. fragilis*.

Effects of cytokines on the production of Th2-attracting chemokines and on the suppressive effects of BB536 on Th2-attracting chemokines

Probiotics induce the production of IL-1 β , IL-6, IL-10, IL-12 and TNF- α by macrophages (He *et al.*, 2002). GM-CSF, IL-4, IL-18 and TNF- α enhance the production of Th2-attracting chemokines by DCs, and IFN- γ and IL-10 suppress it (Fujita *et al.*, 2005). To investigate the mechanism of the production of Th2-attracting chemokines and of the

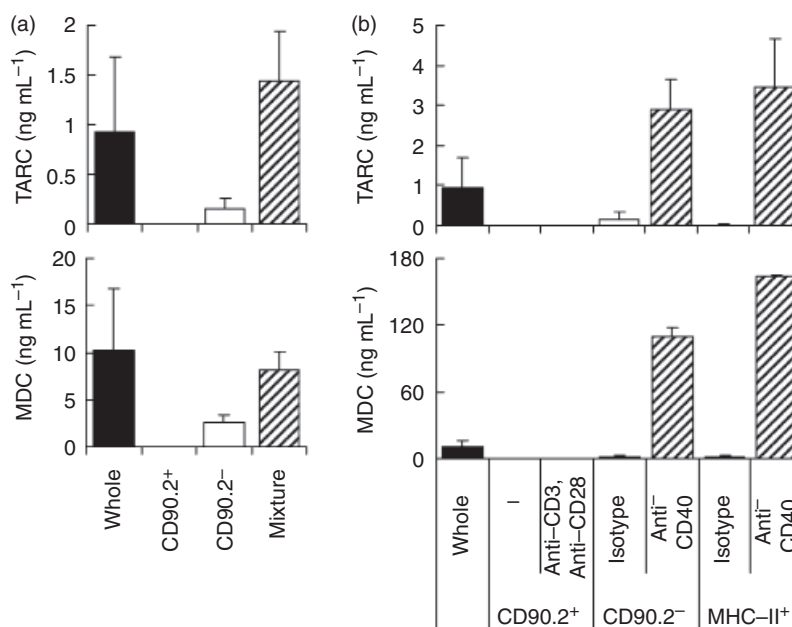


Fig. 2. Interactions with T cells induce the production of Th2-attracting chemokines by APCs. (a) Splenocytes (whole), CD90.2⁺ splenocytes, CD90.2⁻ splenocytes and a mixture of CD90.2⁺ cells and CD90.2⁻ cells (1 : 1) were cultured for 7 days. (b) Splenocytes (whole), CD90.2⁺ cells, CD90.2⁻ cells and MHC class II⁺ cells were cultured with or without (–) the indicated antibodies for 7 days. The levels of TARC and MDC in the supernatants were measured using ELISA. Data are shown as mean $\pm$ SD of three independent experiments.

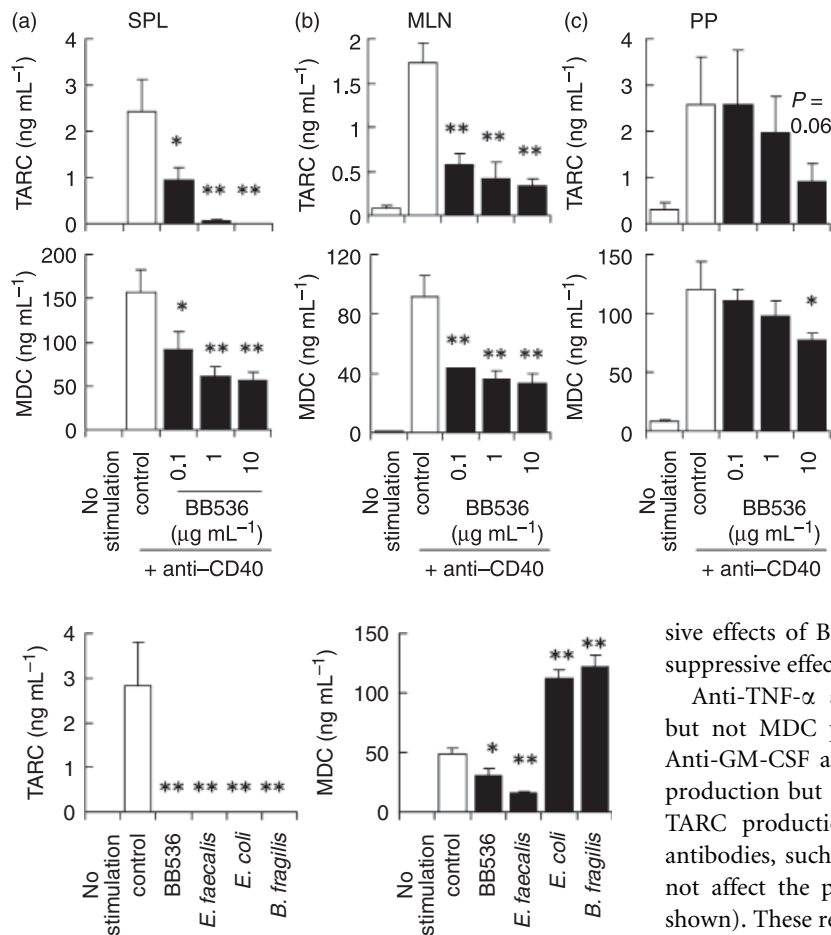


Fig. 3. BB536 suppresses the production of Th2-attracting chemokines by CD90.2⁺ cells from the spleen, MLNs and PPs. CD90.2⁺ cells from the spleen (a), MLNs (b) and PPs (c) were cultured with or without (control) BB536 (0.1–10 $\mu\text{g mL}^{-1}$) in the presence of anti-CD40 antibodies or isotype-matched control antibodies (no stimulation). The levels of TARC and MDC in the supernatant on day 7 were measured using ELISA. Data are shown as mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control.

Fig. 4. Effects of different bacteria on the production of Th2-attracting chemokines by CD90.2⁺ cells. CD90.2⁺ splenocytes were cultured with or without (control) bacteria (10 $\mu\text{g mL}^{-1}$) in the presence of anti-CD40 antibodies or isotype-matched control antibodies (no stimulation). The levels of TARC and MDC in the supernatant on day 7 were measured using ELISA. Data are shown as mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control.

suppressive effects of BB536, we performed cytokine neutralization studies. Anti-IL-12 antibodies (5 $\mu\text{g mL}^{-1}$) and anti-IFN- γ antibodies (5 $\mu\text{g mL}^{-1}$) significantly increased the production of TARC and MDC both in the control cultures and in the BB536-added cultures, as compared with the addition of isotype control antibodies (Fig. 5a and b). The suppression of MDC by BB536 was almost completely abrogated by these neutralizing antibodies, but the suppression of TARC was only moderately abrogated. INF- γ production was not detected in BB536-added cultures when neutralized with anti-IL-12 antibodies, and similar results were obtained when these neutralizing antibodies were added at a concentration of 10 $\mu\text{g mL}^{-1}$ (data not shown). These results indicate that Th1-skewing cytokines such as IL-12 and IFN- γ suppressed the production of TARC and MDC and that these cytokines were involved in the sup-

sive effects of BB536 on MDC and partly involved in the suppressive effects on TARC.

Anti-TNF- α antibodies suppressed TARC production, but not MDC production, in control cultures (Fig. 5c). Anti-GM-CSF antibodies did not affect the level of MDC production but almost completely diminished the levels of TARC production in control cultures (Fig. 5d). Other antibodies, such as IL-1 β , IL-4, IL-6, IL-10 and IL-18, did not affect the production of TARC and MDC (data not shown). These results indicate that GM-CSF and TNF- α are involved in TARC production.

We examined whether BB536 induced the production of IL-12p40, IL-12p70 and IFN- γ by CD90.2⁺ cells with or without anti-CD40 antibodies. IL-12p40 and IFN- γ were induced with anti-CD40 antibodies or BB536, and IL-12p70 was induced with BB536 only (Fig. 5e–g). These data indicate that BB536 has the capability to induce Th1-skewing cytokines that suppress the production of Th2-attracting chemokines.

Production of Th2-attracting chemokines by APCs stimulated with anti-CD40 antibodies and GM-CSF

MDC was produced by B cells (B220⁺ cells), macrophages (CD11b⁺ cells) and DCs (CD11c⁺ cells) that were stimulated by anti-CD40 antibodies; however, only a small amount of TARC was produced by B cells and macrophages in response to stimulation with anti-CD40 antibodies (Fig. 6a–c). On the other hand, the addition of GM-CSF induced the production of TARC by B cells and macrophages. DCs that were stimulated with anti-CD40 antibodies produced a small amount of TARC, but this TARC production was lost by the addition of anti-GM-CSF antibodies

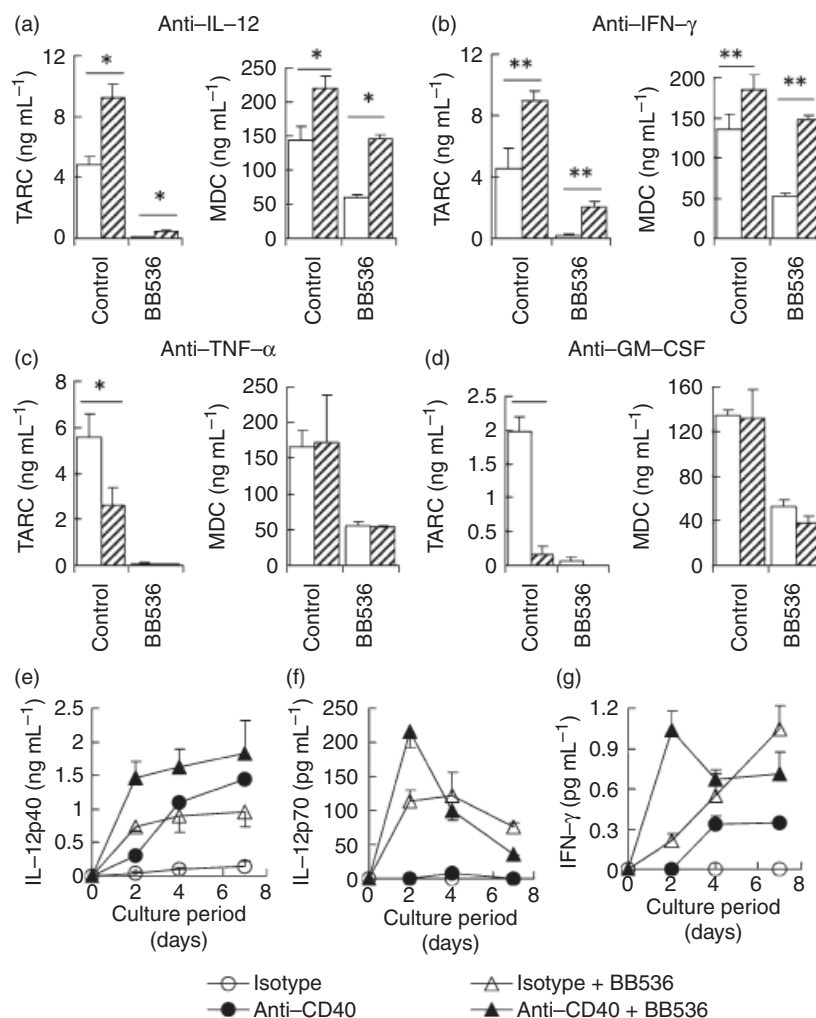


Fig. 5. Cytokine involvement in the suppressive effects of BB536 on Th2-attracting chemokines and in the production of Th2-attracting chemokines. (a–d) CD90.2⁺ splenocytes were cultured with anti-CD40 antibodies (5 µg mL⁻¹) and BB536 (1 µg mL⁻¹) in the presence of neutralizing antibodies (5 µg mL⁻¹, hatched columns) or isotype-matched control antibodies (5 µg mL⁻¹, open columns). The levels of TARC and MDC in the supernatants on day 7 were measured using ELISA. **P* < 0.05, ***P* < 0.01 compared with isotype control. (e–g) CD90.2⁺ splenocytes were cultured with (triangles) or without (circles) BB536 (10 µg mL⁻¹) in the presence of anti-CD40 antibodies (5 µg mL⁻¹, filled symbols) or isotype-matched control antibodies (5 µg mL⁻¹, open symbols) for the indicated period. The levels of IL-12p40 (e), IL-12p70 (f) and IFN-γ (g) in the supernatants were measured using ELISA. Data are shown as mean ± SD of three independent experiments.

(Fig. 6c). MDC production in these DCs was not lost by the addition of anti-GM-CSF antibodies. These results suggest that CD40 signals are crucial for MDC production by APCs and that GM-CSF is crucial for TARC production. BB536 significantly suppressed the production of TARC and MDC under the conditions where these chemokines were clearly induced, but the suppressive effect on MDC production by B cells and DCs was low (Fig. 6a–c). Although TNF-α appeared to be involved in TARC production in the neutralizing experiments, it did not induce TARC production by B cells, macrophages and DCs (data not shown).

GM-CSF production by each population of splenic cells

Because we found that GM-CSF was crucial for TARC production, we studied cells that produce GM-CSF and investigated the effects of BB536 on GM-CSF production (Fig. 7). Whereas whole splenocytes produced some amounts of GM-CSF on day 7, CD90.2⁺ cells alone produced no GM-

CSF; however, CD90.2⁺ cells stimulated with plate-coated anti-CD3 antibodies and addition of anti-CD28 antibodies produced large amounts of GM-CSF. BB536 did not affect this GM-CSF production. CD90.2⁺ cells produced a small amount of GM-CSF only when they were stimulated by anti-CD40 antibodies. DCs produced some GM-CSF, but B cells and macrophages produced only scarce amounts of GM-CSF, regardless of being stimulated by anti-CD40 antibodies. GM-CSF production by DCs was increased, but not significantly, with anti-CD40 antibody stimulation. BB536 slightly increased the GM-CSF production by CD90.2⁺ cells and DCs with CD40 stimulation. These results indicate that GM-CSF was produced by DCs and T cells, with T cells responsible for the greatest amount of GM-CSF, and that BB536 did not suppress the GM-CSF production.

Discussion

Probiotics have been shown to promote Th1 functions and to suppress IgE production and Th2 responses (Shida *et al.*,

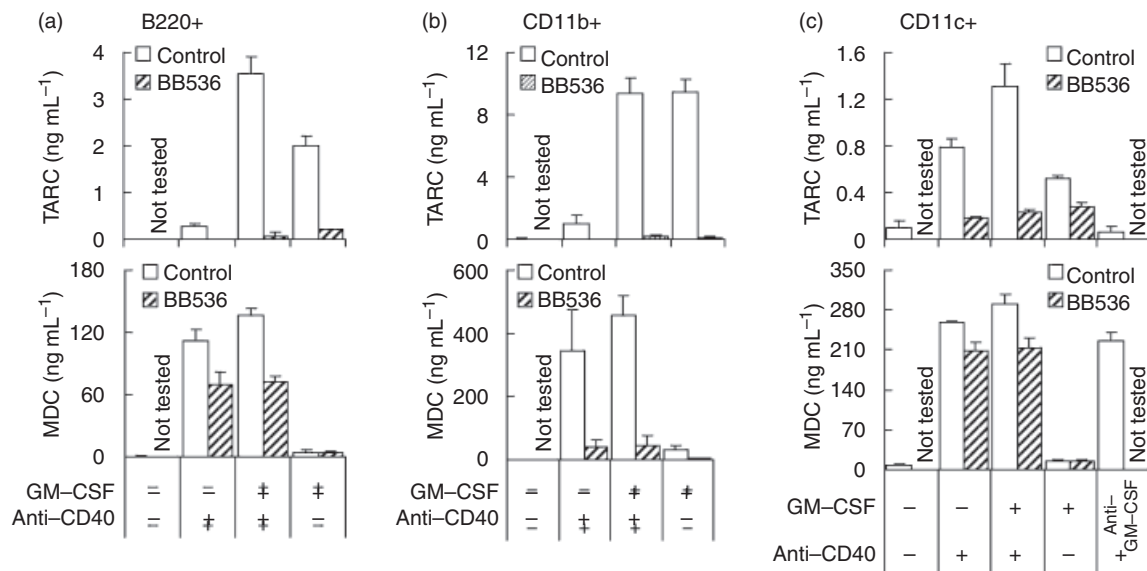


Fig. 6. TARC production by APCs was induced with GM-CSF, and MDC production was induced with a CD40-mediated signal. (a) B220⁺ cells, (b) CD11b⁺ cells and (c) CD11c⁺ cells from the spleen were cultured with or without anti-CD40 antibodies and GM-CSF (10 ng mL⁻¹) in the presence or absence of BB536 (10 µg mL⁻¹). In the case of CD11c⁺ cells, cultures containing anti-CD40 antibodies and anti-GM-CSF antibodies (5 µg mL⁻¹) instead of GM-CSF were also prepared. The levels of TARC and MDC in the supernatants on day 7 were measured using ELISA. Data are shown as mean ± SD of three independent experiments.

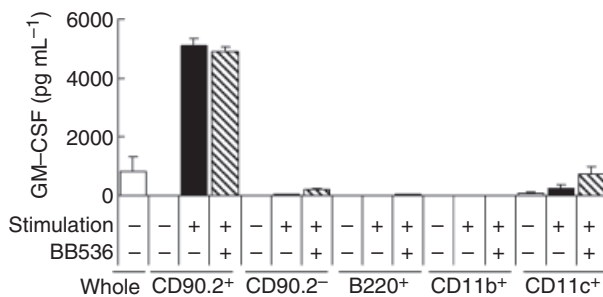


Fig. 7. GM-CSF production by each splenic cell population. Cells indicated in the figure were cultured for 7 days with or without stimulation in the presence or absence of BB536 (10 µg mL⁻¹). CD90.2⁺ cells were stimulated with both plate-coated anti-CD3 antibodies and addition of anti-CD28 antibodies, and CD90.2⁻ cells, B220⁺ cells, CD11b⁺ cells and CD11c⁺ cells were stimulated with addition of anti-CD40 antibodies. The levels of GM-CSF in the supernatants were measured using ELISA. Data are shown as mean ± SD of three independent experiments.

1998; Fujiwara *et al.*, 2004; Mohamadzadeh *et al.*, 2005). Although BB536 suppressed *in vitro* IgE production and Th2 responses, the capability of *Bifidobacterium* strains including BB536 to induce Th1-skewing cytokines was lower than that of lactic acid bacteria such as lactobacilli and streptococci (Iwabuchi *et al.*, 2007). In a human trial on Japanese cedar pollinosis, oral intake of BB536 alleviated subjective symptoms and significantly suppressed the increase in TARC levels associated with pollen dispersion (Xiao *et al.*, 2006b, 2007a). Therefore, we expected that

other mechanisms, besides suppression of IgE production or Th2 responses, could be involved in the antiallergic effects exerted by probiotics. Thus, we investigated the effects of BB536 on the production of Th2-attracting chemokines, such as TARC and MDC.

In the present study, we showed that TARC and MDC were produced by murine splenocytes without stimulation and that BB536 suppressed this production (Fig. 1). These chemokines were produced by APCs of splenocytes and were induced by the T cell–APC interaction (Fig. 2). TARC production was induced with the GM-CSF that was produced by T cells and to a lesser extent by DCs (Figs 6 and 7). On the other hand, MDC production was induced only with CD40 stimulation of the T cell–APC interaction (Fig. 6). These results indicate that T cell–APC interactions are crucial for the production of Th2-attracting chemokines, such as TARC and MDC, by APCs and that BB536 suppresses the *in vitro* production of these chemokines (Figs 3 and 6).

Kuroda *et al.* (2001) have reported that MDC is produced by murine splenic APCs, such as B cells, macrophages and DCs, stimulated with anti-CD40 antibodies; however, there has been no previous report on the production of TARC by murine splenocytes. We supposed that the splenocytes responsible for TARC secretion would be APCs that were induced to secrete TARC by T cell–APC interactions via the CD40 signal. Schaniel *et al.* (1999) have reported that murine B cells activated with anti-CD40 antibodies and murine DCs express TARC mRNA and MDC mRNA.

However, when we measured these chemokines by ELISA, we found that TARC and MDC were produced by MHC class II⁺ cells stimulated with anti-CD40 antibodies, but B cells and macrophages alone, which are the main types of APCs, did not abundantly secrete TARC when stimulated with anti-CD40 antibodies (Figs 3 and 6). Furthermore, taking into consideration the population of DCs in splenocytes, although DCs stimulated with anti-CD-40 antibodies produced a small amount of TARC, the levels of the production by the DCs were low as compared with that by MHC class II⁺ cells (Fig. 6c). These data suggest that factors other than the CD40 signal are necessary for the TARC produced by B cells, macrophages and DCs to reach the level produced by whole splenocytes.

We performed neutralizing studies to elucidate the indispensable cytokines for TARC production, and we found that GM-CSF was involved in TARC production (Fig. 5d). CD14⁺ monocytes isolated from human peripheral blood mononuclear cells abundantly produced TARC with GM-CSF treatment (Imai *et al.*, 1999), and splenic DCs treated with GM-CSF produced TARC (Fujita *et al.*, 2005). In the present study, we found that GM-CSF markedly induced TARC production by B cells and macrophages, and that TARC production by DCs stimulated with anti-CD40 antibodies was diminished by neutralizing GM-CSF (Fig. 6c). CD90.2⁺ cells stimulated with anti-CD40 antibodies and DCs produced GM-CSF, which appears to contribute to the TARC production in cultures of CD90.2⁺ cells or MHC-II⁺ cells. Importantly, T cells produced a high level of GM-CSF in the presence of both anti-CD3 and anti-CD28 (Fig. 7). This finding suggests that GM-CSF mainly produced by T cells is crucial for the TARC production by APCs that is induced with the T cell-APC interaction. We found that MDC production by B cells, macrophages and DCs was induced only with CD40 stimulation of the T cell-APC interaction, similar to the results reported by Kuroda *et al.* (2001). Interaction with T cells induced the production of Th2-attracting chemokines such as TARC and MDC by APCs; this result suggests that T cells accumulated in inflammatory regions cause a vicious circle of accumulation of Th2 cells and aggravate the symptoms of allergic disorders.

BB536 suppressed the production of TARC and MDC by APCs of murine splenocytes stimulated with anti-CD40 antibodies (Figs 3 and 6). Neutralization of IL-12 and IFN- γ completely abrogated the suppressive effect of BB536 on MDC production, but only partially abrogated the suppressive effect of BB536 on TARC production (Fig. 5a and b). Furthermore, we found that BB536 suppressed the TARC production by B cells induced with GM-CSF in the presence of anti-IFN- γ antibodies (data not shown). These results suggested that BB536 could inhibit MDC production through the induction of IL-12 and IFN- γ , but the suppres-

sive effect on TARC production was only partly dependent on these cytokines. BB536 did not affect the GM-CSF production by T cells and slightly increased the GM-CSF production by DCs (Fig. 7), and BB536 suppressed the TARC production by each subset of APCs in the presence of GM-CSF (Fig. 6). These results indicate that BB536 suppresses the TARC production by APCs induced with GM-CSF and there is a possibility that the suppression of TARC production could be due to the direct interaction of BB536 with APCs. Toll-like receptor (TLR) ligands, such as a strain of gram-positive bacterium *Staphylococcus aureus* Cowan 1 (TLR2 ligand), lipopolysaccharide (TLR4 ligand) and CpG (TLR9 ligand), suppress TARC production by murine Langerhans cells (Mitsui *et al.*, 2004). Immunostimulatory oligodeoxynucleotide that is recognized by TLR9 has been identified from the genomic DNA of BB536 (Takahashi *et al.*, 2006a,b,c). Peptidoglycans of cell wall components that are TLR2 ligands and oligodeoxynucleotides of BB536 may possibly suppress TARC production via such TLRs on APCs, but further investigations are needed.

The suppressive effects of BB536 on the production of Th2-attracting chemokines by APCs from the spleen and MLN were superior to those from PPs (Fig. 3). PPs are the predominant sites of antigen sampling, and intestinal immunocompetent cells in PPs interact directly with intestinal bacteria after transport of the bacteria through M cells. Intestinal macrophages and DCs possess functions of inducing a noninflammatory environment to preserve gut immune homeostasis with various mechanisms (Stagg *et al.*, 2003; Smith *et al.*, 2005). Human gut DCs were unable to release IL-12 even in the presence of bacterial stimulation (Rimoldi *et al.*, 2005), and murine intestinal macrophages did not produce proinflammatory cytokines IL-12 and IL-23, but produced large amounts of IL-10 upon stimulation with bacteria (Kamada *et al.*, 2005). It is also known that macrophages exposed to bacterial stimuli, such as lipopolysaccharide and lipoteichoic acid (TLR2 ligand), show hyporesponsiveness to subsequent stimulation with lipopolysaccharide or lipoteichoic acid, termed cross-tolerance (Sato *et al.*, 2000; Lehner *et al.*, 2001). Thus, the low ability to produce IL-12 by intestinal immunocompetent cells, and cross-tolerance induced after repeated contact with components of gram-negative or gram-positive bacteria in PPs, might contribute to the low response to BB536 stimulation on the production of Th2-attracting chemokines in PPs, but further investigation is needed.

Enterococcus faecalis, *E. coli* and *B. fragilis*, similar to BB536, suppressed TARC production by APCs stimulated with anti-CD40 antibodies (Fig. 4). This result suggests the potential of other gram-positive and gram-negative bacteria in the suppression of TARC production, although conclusion awaits further studies on more strains. On the other hand, although MDC production was suppressed by BB536

and *E. faecalis*, MDC production was, in contrast, increased by *E. coli* and *B. fragilis*. Because lipopolysaccharide has been reported to promote MDC production by APCs (Imai *et al.*, 1999), lipopolysaccharide in the cell wall of gram-negative bacteria might contribute to the increased MDC production. It has been shown that, after oral feeding of fluorescent-tagged lactobacilli to mice, fluorescence was detected in immune cells in PPs and the lamina propria in the small intestine as well as in the immune cells in the crypt and lymph nodes in the colon (Delseigneurie *et al.*, 2008). Although the suppressive effects of BB536 on TARC production by APCs from PPs were inferior to those from spleen and MLN, BB536 tended to suppress TARC production by APCs from PPs (Fig. 3). Because the concentration of bacterial cells in the small intestine is much lower than that in the large intestine (Blaut & Clavel, 2007), there is a possibility that administration of bacteria in a large dose such as oral intake of fermented foods could affect the host immune system in the small intestine in preference to normal resident bacteria. As shown in the present study, it is not surprising to find that some commensal bacteria such as *E. faecalis*, *E. coli* and *B. fragilis* showed some *in vitro* activities in the host immune system; however, some extra effects *in vivo* on the host immune response should be expected when some of these bacteria, for example, the probiotics *B. longum* BB536, are orally ingested. Many probiotic strains, most of them gram-positive bacteria, have been demonstrated for antiallergic activity from *in vitro*, *in vivo* as well as human studies (Shimada *et al.*, 2004; Ogawa *et al.*, 2006; Feleszko *et al.*, 2007). We propose that, similar to BB536, suppression of Th2-attracting chemokines may be a part of the mechanisms in the antiallergic activity of some, if not all, probiotics.

Plasma TARC and MDC levels have been significantly correlated with the severity scoring of atopic disease (Fujisawa *et al.*, 2002; Horikawa *et al.*, 2002). In our human trial on Japanese cedar pollinosis, oral intake of BB536 suppressed the aggravation of subjective symptom scores and the increase of TARC levels associated with pollen dispersion, and significant positive correlations were found between elevated values of TARC and symptom scores recorded in the pollen season (Xiao *et al.*, 2006b, 2007a). Moreover, BB536 intake reduced some of the delayed symptoms and the prevalence of medication use after exposures to antigen in a trial performed in an environmental exposure unit (Xiao *et al.*, 2007b). Considering that Th2-attracting chemokines play a crucial role in the late inflammatory reaction, we speculated that the alleviation of subjective symptoms of Japanese cedar pollinosis by BB536 might be mediated by the suppression of TARC production. The results from the present study support the clinical findings of BB536 in suppressing Th2-attracting chemokines and suggest a novel mechanism of BB536 in the

antiallergic effects besides suppression of IgE production or Th2 responses.

In conclusion, we demonstrated that heat-killed BB536 suppressed the production of Th2-attracting chemokines induced by the T cell–APC interaction. This result suggests a novel mechanism for alleviating symptoms of allergic disorders by probiotics. Because BB536 suppressed the production of Th2-attracting chemokines by APCs not only from the spleen but also from MLNs and PPs (Fig. 3), oral administration of BB536 is expected to suppress these productions *in vivo*. However, further studies to elucidate the differences between dead bacteria and live bacteria of BB536 on TARC production and the *in vivo* involvement of Th2-attracting chemokines in the antiallergic effects of probiotics with animal models are needed.

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