BASIC RESEARCH

Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells

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Abstract

AIM: To analyze the ability of nine different potentially probiotic bacteria to induce maturation and cytokine production in human monocyte-derived dendritic cells (moDCs).

METHODS: Cytokine production and maturation of moDCs in response to bacterial stimulation was analyzed with enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis (FACS), respectively. The kinetics of mRNA expression of cytokine genes was determined by Northern blotting. The involvement of different signaling pathways in cytokine gene expression was studied using specific pharmacological signaling inhibitors.

RESULTS: All studied bacteria induced the maturation of moDCs in a dose-dependent manner. More detailed analysis with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH74 indicated that these bacteria induced the expression of moDC maturation markers HLA class II and CD86 as efficiently as pathogenic bacteria. However, these bacteria differed in their ability to induce moDC cytokine gene expression. *S. thermophilus* induced the expression of pro-inflammatory (TNF-α, IL-12, IL-6, and CCL20) and Th1 type (IL-12 and IFN-γ) cytokines, while *B. breve* and *L. lactis* were also potent inducers of anti-inflammatory IL-10. Mitogen-activated protein kinase (MAPK) p38, phosphatidylinositol 3 (PI3) kinase, and nuclear factor-kappa B (NF-κB) signaling pathways were shown to be involved in bacteria-induced cytokine production.

CONCLUSION: Our results indicate that potentially probiotic bacteria are able to induce moDC maturation, but their ability to induce cytokine gene expression varies significantly from one bacterial strain to another.

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INTRODUCTION

The importance of the intestinal microbiota, and the ways in which it can be modified, has been under intensive investigation in recent years. Evidence from randomized and placebo-controlled clinical trials and molecular studies has attracted significant interest to the use of probiotic bacteria as therapeutic substances. Probiotics are live microbial food supplements that have a beneficial effect on human health. They can

change the composition or metabolic activity of the intestinal microbiota and modulate the immune system of the host. This seems to be dependent on the probiotic bacterial species used and the overall health status of the host[1]. Probiotic bacteria have been used as an alternative form of therapy for various diseases and symptoms[2,3]. Probiotic supplement containing *L. rhamnosus* GG, *L. rhamnosus* LC705, *P. freudenreichii* JS, and *B. breve* Bb99 alleviated the symptoms of irritable bowel syndrome (IBS) and improved eradication of *H pylori*[4,5]. The best studied probiotic bacterium, *L. rhamnosus* GG has been found to be effective in the treatment of antibiotic-associated[6] and nosocomial diarrhea[7] in children. In addition, *L. rhamnosus* GG has been used to reduce the onset of atopy[8]. It has also been shown to have immunomodulatory effects on the host by decreasing the production of IL-6 and TNF-α in healthy individuals[9] and TNF-α production in allergic children[10]. All bacteria used in the present work are Gram-positive bacteria and some of them are widely used in the food industry. *S. thermophilus* THS, *L. mesenteroides* subsp. *cremoris* PIA2, and *L. lactis* subsp. *cremoris* ARH74 are used for the fermentation of milk products. *L. rhamnosus* GG and *B. animalis* subsp. *lactis* Bb12 are used as supplements in dairy products due to their proved probiotic effects. *B. animalis* subsp. *lactis* Bb12 is shown to be effective in reducing the risk of diarrhea and modulating the immune response[11,12]. Although many probiotic bacterial strains are used as food supplements, their mechanisms of action have remained poorly characterized.

The gut is rich in antigen-presenting cells such as dendritic cells (DCs), which reside underneath the epithelial cell layer in an immature state being constantly on alert for foreign antigens or invading pathogens[13,14]. DCs can reach their dendrites through the tight junctions of epithelial cells and in this manner sample the gut for different antigens[14,15]. Upon contact with microbes or foreign antigens, DCs undergo a maturation process, which is associated with the expression of cell surface costimulatory molecules CD80, CD83, and CD86 and migration from the peripheral tissues into local lymph nodes. In the lymph nodes mature DCs present antigens to naive T cells, and the development of adaptive immune responses is initiated[16-18]. Depending on the nature of the antigen, the magnitude of the activation of innate and adaptive immune responses can vary greatly. Cytokine production profile and the maturation status of DCs depend on the nature of the stimulus and determine whether T cells are polarized towards T helper (Th) 1, Th2, or T regulatory (Treg) type responses[19-21].

DCs have multiple receptor systems including scavenger receptors, lectin-binding molecules, Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain (NOD) molecules that recognize different types of bacterial structural components or genetic material[22-24]. Activation of DCs *via* TLRs leads to the activation of intracellular signal transduction pathways and to the activation and nuclear translocation of NF-κB, interferon regulatory factor (IRF), or mitogen-activated protein kinase (MAPK)-regulated transcription factors, which ultimately regulate the activation status of DCs and enhance the expression of cytokine genes[25-27]. However, it still remains elusive how DCs can differentiate between commensal and pathogenic bacteria.

In the present study we have analyzed the ability of nine different, potentially probiotic bacteria to induce maturation and cytokine gene expression in human moDCs. By analyzing the cytokine production profiles in moDCs induced by these bacteria, we hope to gain insight into different immunomodulatory effects of probiotic bacteria. This information is of value for selecting new probiotics for *in vivo* trials.

MATERIALS AND METHODS

Bacterial strains

Two well-known probiotic strains *L. rhamnosus* GG (ATCC 53103)[7,10] and *B. animalis* subsp. *lactis* Bb12 (DSM15954)[12], and seven potentially probiotic strains *L. rhamnosus* LC705 (DSM 7061)[4,5], *L. helveticus* 1129 (DSM 13137)[28], *B. longum* 1/10, *B. breve* Bb99 (DSM 13692)[4,5], *S. thermophilus* THS[29,30], *L. lactis* subsp. *cremoris* ARH74 (DSM 18891)[31], and *L. mesenteroides* subsp. *cremoris* PIA2 (DSM 18892)[29] were obtained from Valio Research Centre (Helsinki, Finland). Pathogenic *S. pyogenes* serotype T1M1 (IH32030)[32], isolated from a child with bacteremia, was from the collection of National Public Health Institute (Helsinki, Finland), and was used as a positive control[33,34]. Bacteria were stored in skimmed milk at -70℃ and grown to the end of logarithmic growth phase before they were used in experiments. *S. pyogenes* and *L. rhamnosus* GG were grown as previously described[32,34]. All strains were passaged three times, except for the Bifidobacterium strains which were passaged four times, before they were used in stimulation experiments. The number of bacteria was determined by counting them in a Petroff-Hausser chamber.

Frozen stocks of *L. rhamnosus* GG, *L. rhamnosus* LC705, and *L. mesenteroides* subsp. *cremoris* PIA2 strains were inoculated in deMan, Rogosa, and Sharpe (MRS) medium (Lab M, Topley House, Lancashire, UK) and grown under aerobic conditions at 37℃. *L. helveticus* 1129 was grown at 42℃ under aerobic conditions in MRS medium (Lab M, Topley House). Bifidobacterium strains were grown anaerobically in MRS (Lab M, Topley House) supplemented with 5 g/L L-cysteine (Merck, Darmstadt, Germany) at 37℃. *S. thermophilus* was grown aerobically at 37℃ on M17 agar (Lab M, Topley House) supplemented with 20 g/L D (+) lactose monohydrate (J.T. Baker B.V., Deventer, Holland), and transferred to M17 broth (Difco, Beckton Dickinson, MD, USA) containing 20 g/L lactose (J.T. Baker B.V.). *L. lactis* was grown on calcium citrate agar (Valio Ltd.) and M17 broth (Difco) containing 20 g/L lactose (J.T. Baker B.V.) at 22℃ under aerobic conditions. For stimulation experiments bacteria were grown to a late logarithmic growth phase, and the indicated amount of bacteria was collected by centrifugation. After this cells were

suspended to RPMI-1640 medium (Sigma, St. Louis, MO, USA) and added to moDC cultures.

DC purification and differentiation

Monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) as described previously[33]. Human peripheral blood mononuclear cells were isolated by density gradient centrifugation over a Ficoll-Pague gradient (Amersham Biotech, Uppsala, Sweden) followed by purification of monocytes in Percoll gradient (Amersham Biotech) centrifugation. Monocytes were collected from the top layer of the gradient, and T and B cells were depleted using anti-CD3 and anti-CD19 magnetic beads (Dynal, Oslo, Norway). Purified monocytes were allowed to adhere to six-well plates 2.5×10^6 cells/well (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for 1 h at 37℃ in RPMI-1640 supplemented with 0.6 μg/mL penicillin (Gibco, BRL, Paisley, Scotland), 60 μg/mL streptomycin (Gibco), 2 mmol/L L-glutamine (Sigma), and 20 mmol/L HEPES. For inhibitor assays, 24-well plates were used (1.25×10^6 cells/well). To differentiate monocytes into immature DCs, they were grown in RPMI-1640 medium (Sigma) supplemented as above, plus 100 mL/L fetal calf serum (FCS) (Integro, Zaandam, Holland), 10 ng/mL recombinant human granulocyte macrophage-colony stimulating factor, GM-CSF (Biosource, Camarillo, CA, USA), and 20 ng/mL recombinant human interleukin (IL)-4 (R&D Systems, Abingdon, UK). Fresh medium was added every 2 d, and the cells were used in experiments after 6 to 7 d of cultivation. Cultured cells were CD1a$^+$, CD14$^-$, CD80$^+$, CD83$^-$, and CD86$^+$ as analyzed by flow cytometry, and they showed a typical DC morphology (data not shown).

Stimulation experiments

All stimulation experiments were carried out with cells obtained from three to four blood donors and conducted in RPMI-1640 medium containing 100 mL/L FCS. MoDCs were stimulated with a bacteria:host cell ratio of 2:1, 10:1, or 40:1. Cells were collected either for flow cytometric analysis (FACS) or for isolation of total cellular RNA. Supernatants were collected and stored at -20℃.

In cytokine priming experiments moDCs were pre-treated with IL-6 (Biosource), TNF-α (Biosource), or IFN-γ (Finnish Red Cross Blood Transfusion Center) for 16 h prior to bacterial stimulation. IL-6 and TNF-α were used at a concentration of 10 ng/mL, and IFN-γ at 100 IU/mL. Supernatants were collected at 24 h after bacterial stimulation for cytokine measurements.

In inhibitor experiments moDCs were treated with different signaling inhibitors for 30 min prior to bacterial stimulation. Five different inhibitors were used at two concentrations: PD98059 (10 μmol/L or 2 μmol/L) and LY294002 (50 μmol/L or 10 μmol/L) both from Calbiochem (San Diego, CA, USA), and cyclosporin A (CsA) (1 μg/mL or 0.2 μg/mL), SB202190 (10 μmol/L

or 2 μmol/L), and pyrrolidine dithiocarbamate (PDTC) (100 μmol/L or 20 μmol/L) all from Alexis Biochemicals (Lausen, Switzerland). Supernatants were collected at 24 h after stimulation, and cytokine levels were determined.

Cytokine-specific enzyme-linked immunosorbent assays (ELISAs)

Cytokine and chemokine levels from cell culture supernatants were analyzed by a sandwich ELISA method as previously described[34]. TNF-α, IL-6, IL-10, and CXCL10 levels were determined by using antibody pairs and standards obtained from BD Pharmingen (San Diego, CA, USA). IL-12p70 and IFN-γ levels were determined with Eli-pair kits (Biosite, Täby, Sweden), and CCL19 and CCL20 levels with Duoset kits (R&D Systems) as instructed by the manufacturer. Cytokine levels in the bacterial dose response experiments were measured using FlowCytomix human Th1/Th2 10plex kit (Bender Medsystems, Vienna, Austria) according to the manufacturer's instructions.

FACS analysis

MoDCs were stimulated with different bacteria for 24 h. Cells were collected, washed once with PBS, and fixed with 10 mL/L paraformaldehyde for 15 min in PBS. After fixation, cells were washed twice with PBS and suspended in PBS + 20 mL/L FCS. For staining of cell surface marker proteins FITC- and PE-conjugated monoclonal antibodies against CD86, HLA class Ⅱ, and isotype matched control antibodies (Caltag Laboratories) were used. Paraformaldehyde-fixed moDCs were stained with mAbs for 30 min at 4℃. The cells were washed twice with PBS + 20 mL/L FCS, suspended in the same solution and analyzed with FACScan flow cytometric device using CellQuest software (Becton Dickinson).

In inhibitor experiments the viability of the cells was confirmed by dead cell discriminator (DCD) (Caltag Laboratories, Burlingame, CA, USA), containing propidium iodide (PI). PI integrates with DNA in non-viable cells with compromised membranes and can be detected by flow cytometry. DCD was added to a suspension of non-fixed cells, 5 μL of DCD per 10^6 cells, immediately prior to FACS, performed as previously described[35].

RNA isolation and Northern blot analysis

For RNA analysis, cells were collected, washed once with PBS, and total cellular RNA was isolated with Rneasy Midi kit (Qiagen). RNA was quantified spectrophotometrically (A_{260nm}) and samples containing equal amounts of RNA (10 μg) were separated by size on 1% formaldehyde-agarose gels and transferred to hybond-N-membranes (Amersham Pharmacia Biotech). To control equal RNA loading, ethidium bromide staining was used. The following human cytokine cDNA probes were used for hybridization: CCL20[36], TNF-α (American Type Culture Collection, Manassas, VA), IL-12p35[33], IFN-γ[37], and IL-10 (DNAX, Palo Alto, CA, USA).

Table 1 Bacteria used in the study

Bacterial species/subspecies	Strain abbreviation	ATCC/DSM number	Ref.	Use in dairy products
Streptococcus				
Streptococcus pyogenes	GAS	NA	[32]	NA
Streptococcus thermophilus	THS	NA	[29,30]	Yoghurt
Lactobacillus				
Lactobacillus rhamnosus GG	LGG	ATCC 53103	[10,7]	Probiotic supplement
Lactobacillus rhamnosus LC705	LC705	DSM 7061	[4,5]	Cheese
Lactobacillus helveticus	1129	DSM 13137	[28]	Cheese, fermented milk
Lactococcus				
Lactococcus lactis subsp. *cremoris*	ARH74	DSM 18891	[31]	Sour milk production
Leuconostoc				
Leuconostoc mesenteroides subsp. *cremoris*	PIA2	DSM 18892	[29]	Sour milk production
Bifidobacterium				
Bifidobacterium animalis subsp. *lactis*	Bb12	DSM 15954	[12]	Probiotic supplement
Bifidobacterium breve	Bb99	DSM 13692	[4,5]	NA
Bifidobacterium longum	1/10	NA	NA	NA

NA: Not applicable.

The probes were labelled with [α-^{32}P] deoxy-adenosine 5'-triphosphate (3000 Ci/mmol, Amersham Pharmacia Biotech) using a random-primed DNA labelling kit (Fermentas, Burlington, Ontario, Canada). Membranes were hybridized o/n in Ultrahyb buffer (Ambion, Austin, TX, USA) at 42℃. Membranes were washed three times with 1 g/L SDS in 1 × saline sodium citrate at 42℃ for 30 min and once at 65℃ for 30 min. Membranes were exposed to Kodak X-Omat AR films (Eastman Kodak, Rochester, NY, USA) at -70℃ with intensifying screens.

RESULTS

Bacteria-induced cytokine production in human moDCs

MoDCs were stimulated with different doses of probiotic bacteria to determine the optimal bacterial dose for further stimulation experiments (Figure 1). The bacteria used in the present study are summarized in Table 1. *S. thermophilus* THS efficiently induced TNF-α, IL-6, and IL-12 production. The two Bifidobacterium strains, *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99, were potent inducers of all measured cytokines TNF-α, IL-1β, IL-6, IL-10, IL-12, and IFN-γ. In contrast, *B. longum* strain 1/10 was not as efficient as *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99 in inducing cytokine, production. *L. lactis* subsp. *cremoris* ARH74 and *L. helveticus* 1129 were as good as bifidobacteria at inducing cytokine production in moDCs. *L. rhamnosus* strains GG and LC705 as well as *L. mesenteroides* subsp. *cremoris* PIA2 were poor inducers of cytokine production and stimulation of moDCs with these bacteria did not result in increased cytokine production compared to unstimulated cells. There was a direct correlation between bacterial dose and cytokine response. Bacteria: host cell ratio of 40:1 showed the highest cytokine production levels (Figure 1).

All studied bacteria induced CCL20 production in a dose-dependent manner (Figure 2), while none of the bacteria were able to induce CCL19 production. *S. thermophilus* was the most efficient probiotic bacterium in inducing CXCL10 production in moDCs. The dose-dependent responses demonstrated that the bacteria: host cell ratio of 40:1 was also most effective in inducing moDC chemokine production.

The effects of bacteria on moDC maturation

Since different probiotic bacteria are able to induce variable cytokine and chemokine responses in human moDCs (Figures 1 and 2), we analyzed whether these bacteria would also differ in their ability to induce moDC maturation. Maturation of DCs is characterized by up-regulation of cell surface maturation marker proteins CD80, CD86, and HLA class Ⅱ[38]. For these studies we chose *B. breve* Bb99, *S. thermophilus* THS, and *L. lactis* subsp. *cremoris* ARH74 that represent different genera and were able to stimulate a variety of moDC cytokine responses (Figure 1). MoDCs were stimulated with selected bacteria at bacteria:host cell ratio of 2:1, 10:1, or 40:1. After stimulation the expression of CD86 and HLA class Ⅱ was analyzed by flow cytometry. Cells from different donors were pooled before the analysis. In *S. thermophilus* THS and *B. breve* Bb99 stimulated moDCs highest up-regulation of HLA class Ⅱ was observed with bacteria:host cell ratio of 10:1, whereas with *L. lactis* subsp. *cremoris* ARH74 a ratio of 40:1 was required for maximal HLA class Ⅱ induction (Figure 3A). The highest CD86 expression was seen at bacteria:host cell ratio of 40:1 (Figure 3B) with all studied bacteria. It is noteworthy that even the lowest bacterial dose (2:1) increased the expression of HLA Ⅱ and CD86. MoDCs stimulated with probiotic bacteria matured equally well as cells stimulated with pathogenic *S. pyogenes*, a known inducer of moDC maturation[33]. The majority of the bacteria-stimulated cells were HLA class Ⅱ/CD86 double positive (Figure 3C). Maturation of moDCs was also observed with all other studied bacteria (data not shown).

Kinetics of bacteria-induced cytokine and chemokine mRNA expression

To further compare the cytokine gene expression profiles induced by *S. thermophilus* THS, *B. breve* Bb99,
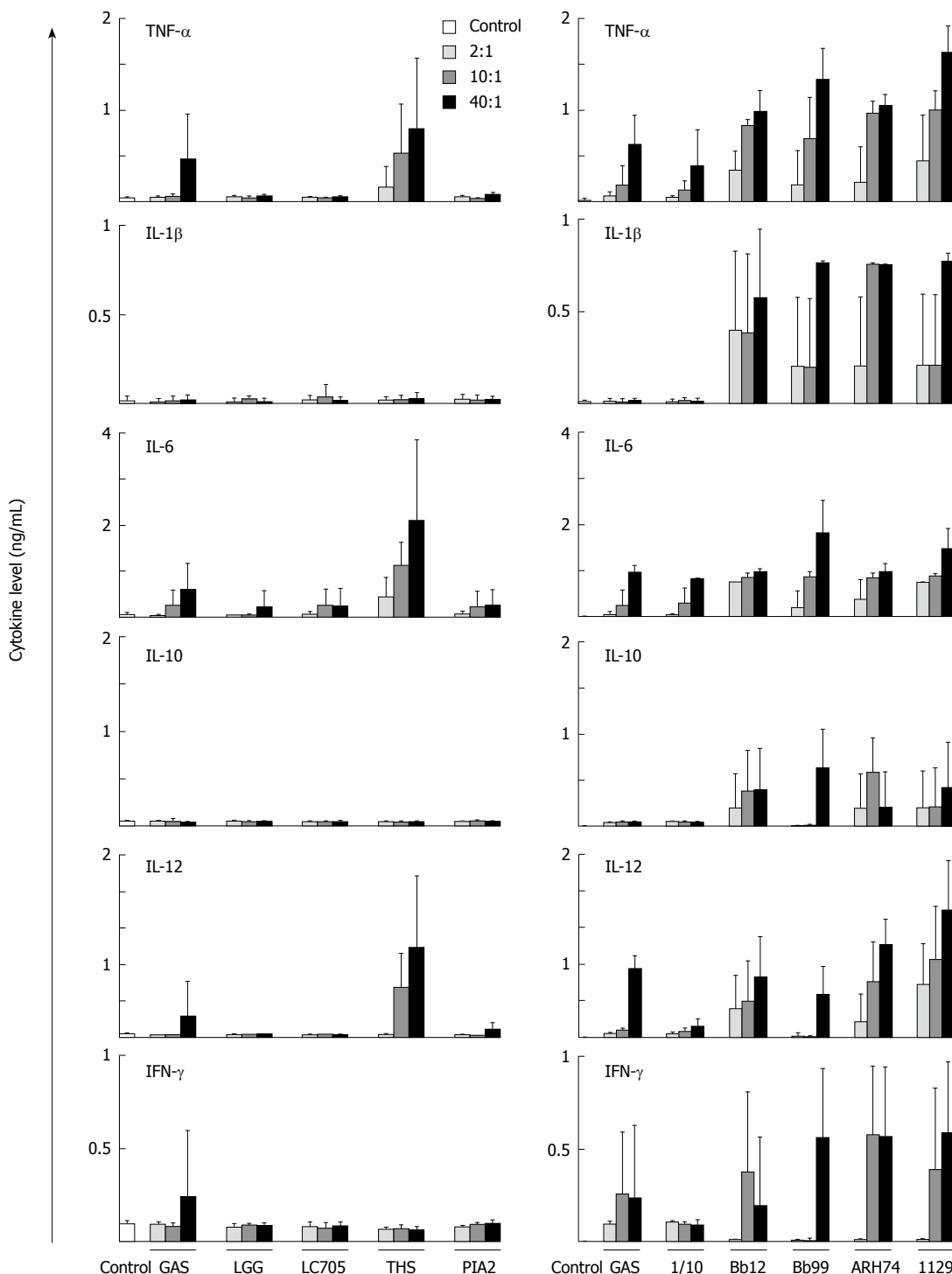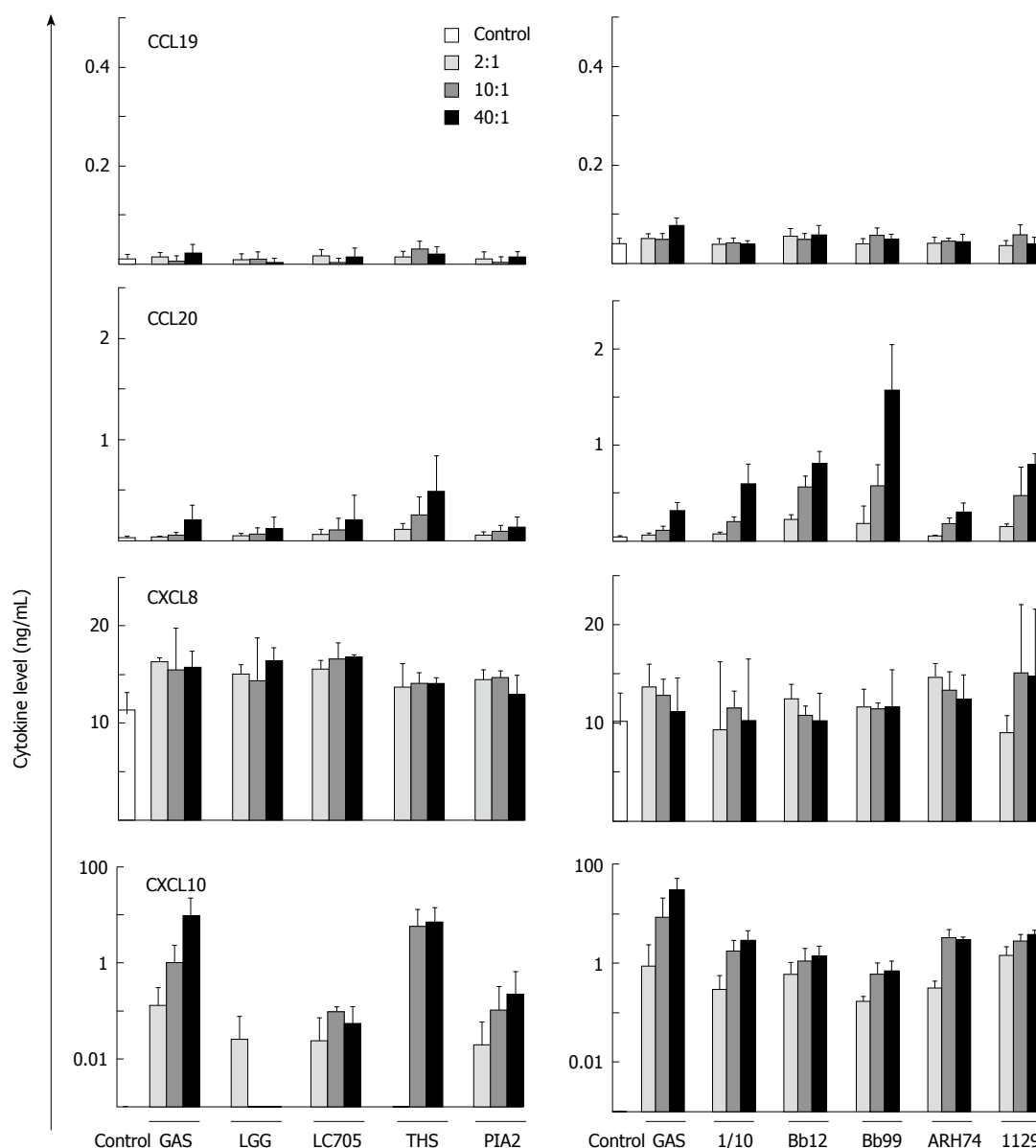
Figure 1 Probiotic bacteria induce cytokine production in human moDCs in a dose-dependent manner. MoDCs were stimulated with bacteria:host cell ratio of 2:1, 10:1, or 40:1. At 24 h after stimulation cell culture supernatants were collected and cytokine levels were determined by ELISA. The experiment was carried out with cells obtained from four different blood donors, and the columns represent means and error bars indicate the standard deviations. The data is from a representative experiment out of two. GAS: *S. pyogenes*; LGG: *L. rhamnosus* GG; LC705: *L. rhamnosus* LC705; THS: *S. thermophilus* THS; PIA2: *L. mesenteroides* subsp. *cremoris* PIA2; 1/10: *B. longum* 1/10; Bb12: *B. animalis* subsp. *lactis* Bb12; Bb99: *B. breve* Bb99; ARH74: *L. lactis* subsp. *cremoris* ARH74; 1129: *L. helveticus* 1129.

and *L. lactis* subsp. *cremoris* ARH74, moDCs were stimulated with these bacteria at a 40:1 bacteria:host cell ratio for 8, 24, or 48 h. Cells from different donors were collected and pooled. Total cellular RNA was isolated for Northern blot analysis (Figure 4A). The mRNA expression of CCL20 and TNF-α was enhanced 8 h after stimulation with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH74. The expression of

IL-12p35 or IFN-γ mRNA was not enhanced by any of the potentially probiotic bacteria. CCL20 mRNA level was highest in *B. breve* Bb99 stimulated cells at 8 h after stimulation. Low levels of CCL20 mRNA were detected in *S. pyogenes* and *S. thermophilus* THS stimulated cells at 8 h after stimulation. CCL20 protein level was highest at the 48 h time point in *B. breve* Bb99 stimulated cells (Figure 4B). TNF-α mRNA expression was strongest in

Figure 2 Probiotic bacteria induce chemokine production in moDCs. MoDCs were stimulated with bacteria:host cell ratio of 2:1, 10:1, or 40:1. Cell culture supernatants from four blood donors were collected at 24 h after bacterial stimulation and the chemokine levels were determined by ELISA. The data is shown as means and error bars indicate standard deviations. Note the differences in scales. The data is from a representative experiment out of two.

B. breve Bb99 stimulated moDCs. In moDCs stimulated with *S. thermophilus* THS and *L. lactis* subsp. *cremoris* ARH74 TNF-α mRNA levels were lower than in *B. breve* Bb99 stimulated moDCs. Cell culture supernatants were also collected and cytokine levels were determined by ELISA. The cytokine production patterns correlated with the kinetics and magnitude of bacteria induced mRNA expression profiles (Figure 4B). IL-12 and IFN-γ mRNAs were not detectable in cells stimulated with probiotic bacteria, and also protein levels remained lower than in *S. pyogenes* stimulated moDCs. IL-10 mRNA and protein expression was best induced by *B. breve* Bb99.

Pharmacological signaling inhibitors affect the cytokine production of probiotic bacteria-stimulated moDCs

In order to obtain insight into the mechanisms of probiotic bacteria-induced cytokine gene expression

in human moDCs, we used different pharmacological inhibitors for MAPK, PI3K, NF-κB, and nuclear factor of activated T cells (NFAT) signaling pathways to study whether they interfere with cytokine production in bacteria-stimulated cells.

B. breve Bb99 was chosen for these experiments due to its ability to effectively induce cytokine production in moDCs. Cells were treated with the inhibitors 30 min prior to bacterial stimulation and the inhibitors were present throughout the stimulation experiment. The p38 MAPK inhibitor SB202190[39] decreased the production of TNF-α, IL-6, IFN-γ, and IL-10 production in a dose-dependent manner (Figure 5). The inhibitor of PI3 kinase, LY294002[40], abolished CXCL10, TNF-α, IFN-γ, and IL-10 production at a concentration of 50 μmol/L. Concentration 100 μmol/L of NF-κB inhibitor PDTC reduced CXCL10 and IFN-γ production, whereas no significant inhibition in the production of other
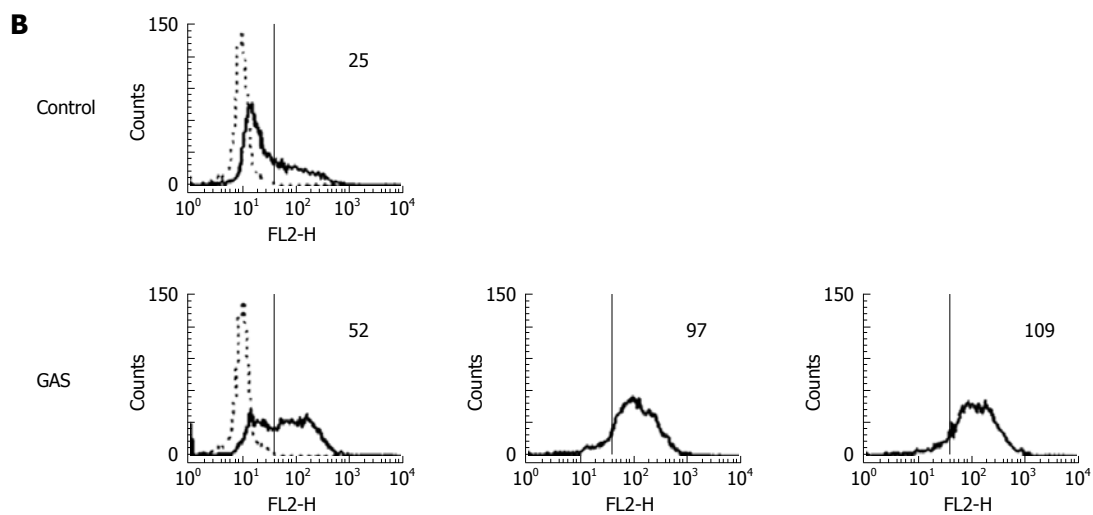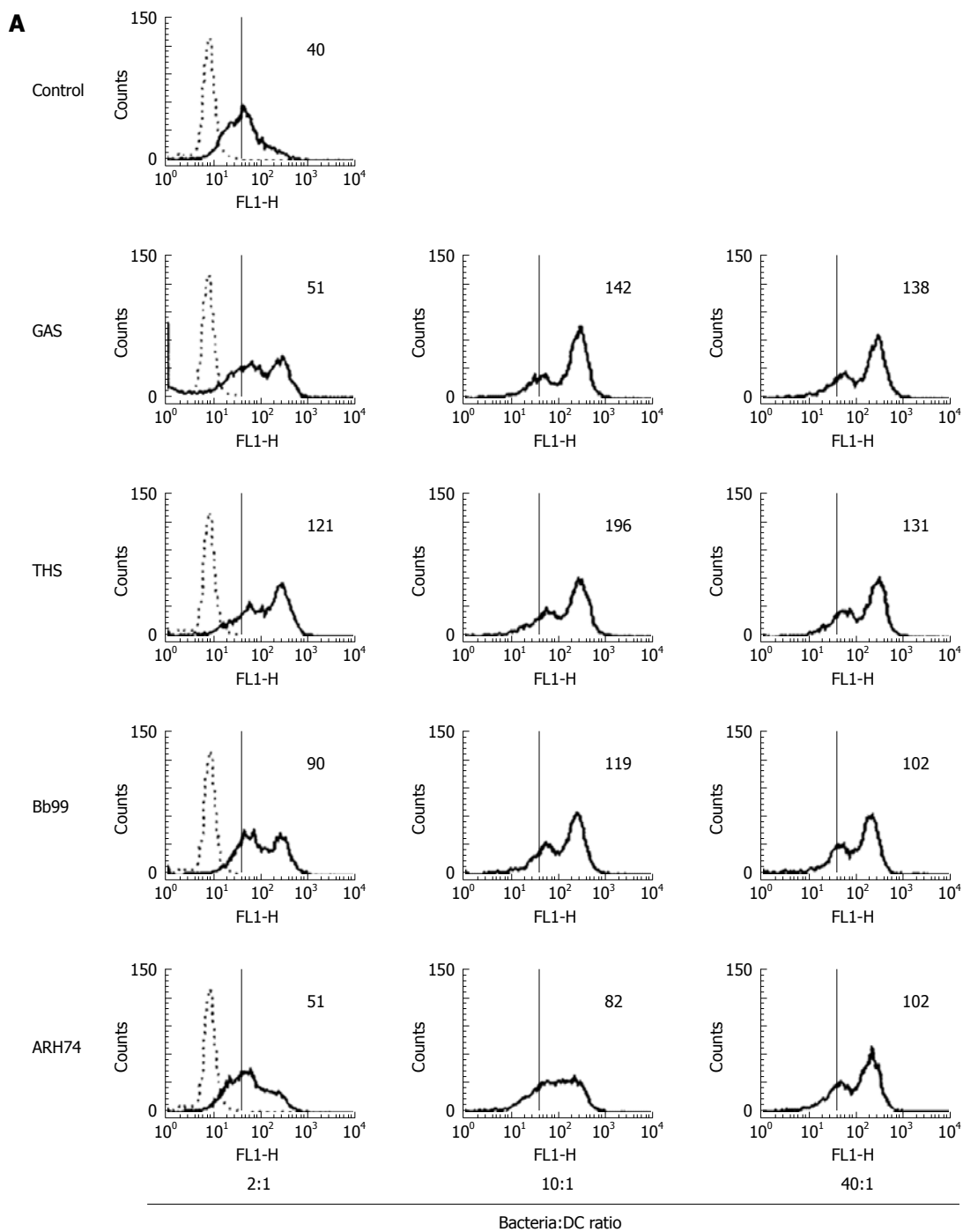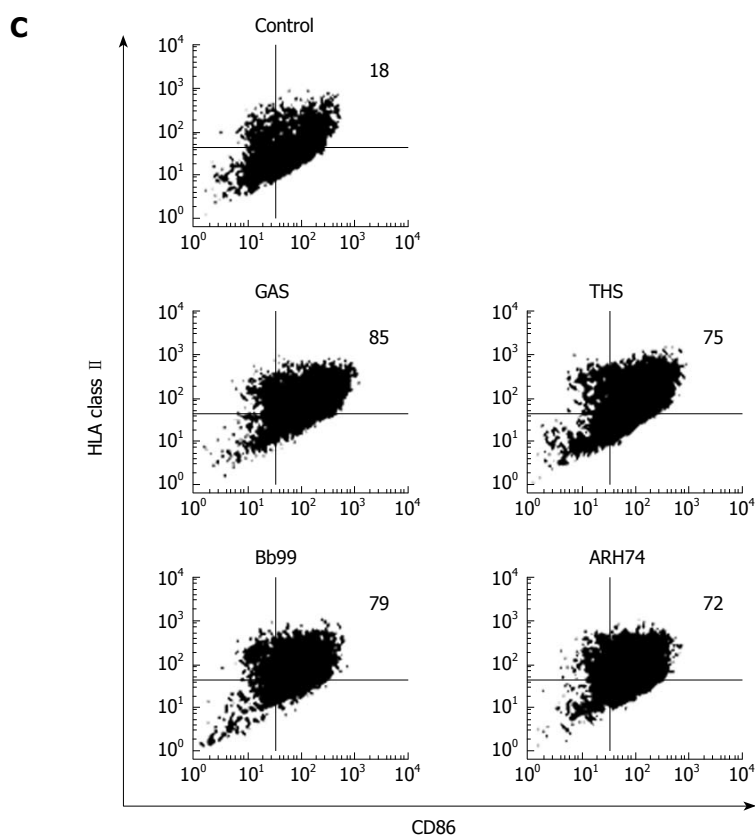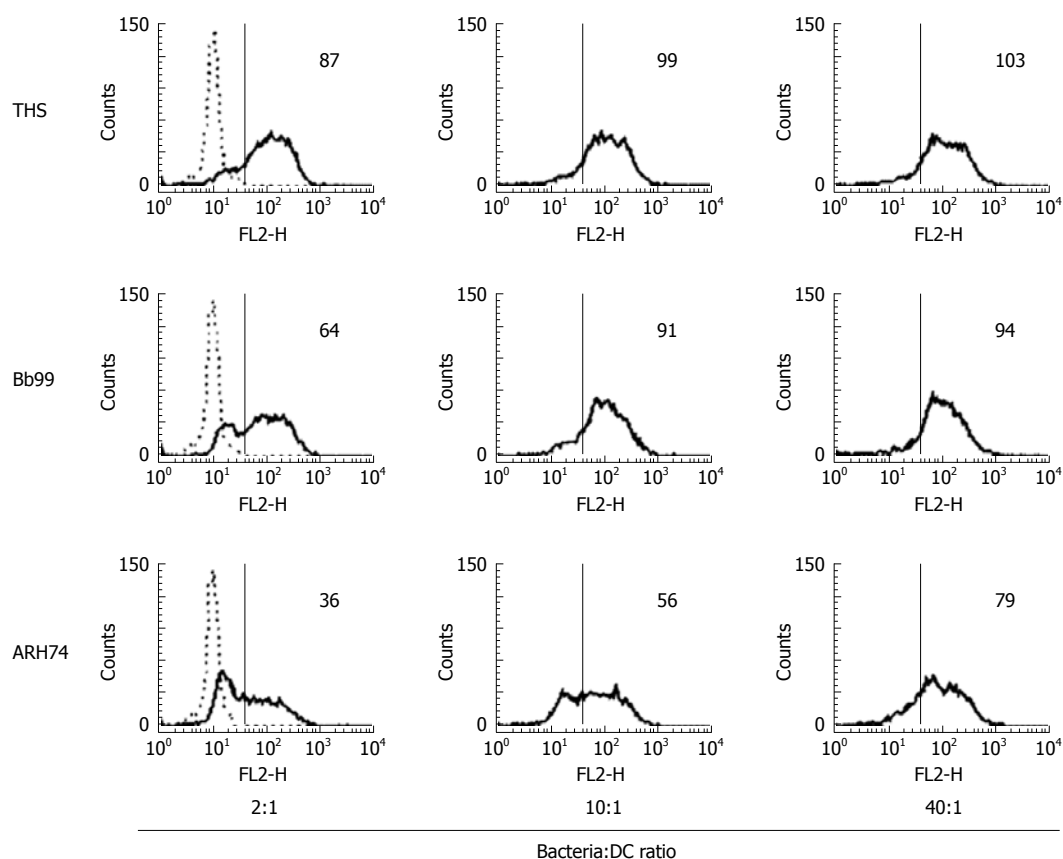
Figure 3 The effect of bacterial dose on the maturation of moDCs. MoDCs were stimulated with bacteria:host cell ratio of 2:1, 10:1, or 40:1. Cells from four different donors were collected, pooled, and stained with antibodies against (**A**) HLA class Ⅱ and (**B**) CD86. The expression of co-stimulatory molecules was analyzed by flow cytometry. The values represent mean fluorescence intensities (MFIs). Results from a representative experiment out of two are shown. Dotted lines indicate respective isotype controls. The staining profiles of HLA class Ⅱ/CD86 double-positive (percentages included in the profiles) cells (**C**). GAS: *S. pyogenes*; THS: *S. thermophilus* THS; Bb99: *B. breve* Bb99; ARH74: *L. lactis* subsp. *cremoris* ARH74.
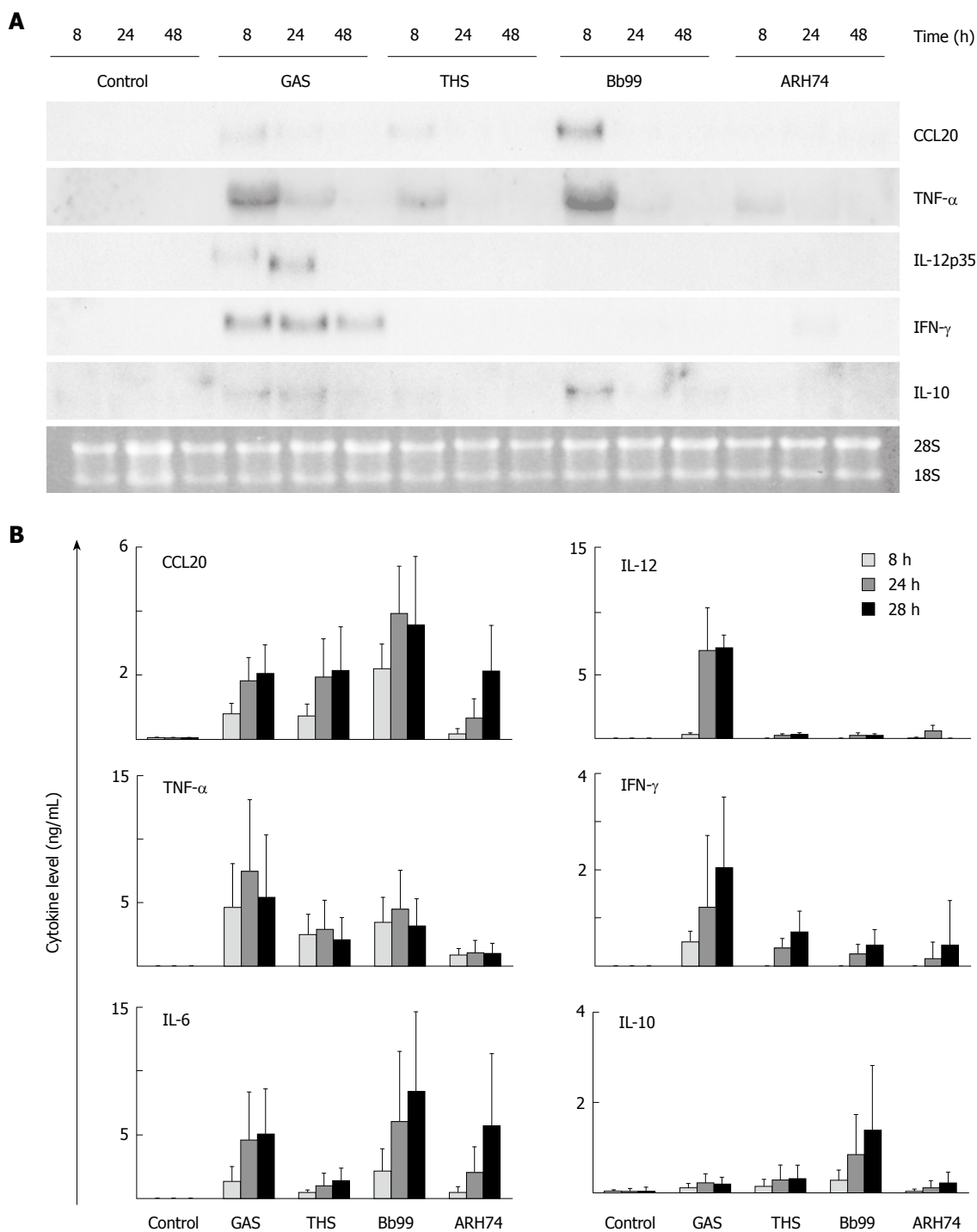
Figure 4 Kinetics of probiotic bacteria induced cytokine gene expression in human moDCs. MoDCs were stimulated with GAS and indicated probiotic bacteria with a bacteria:host cell ratio of 40:1. **A**: Cells were collected at 8, 24, and 48 h after stimulation and total cellular RNA was isolated. mRNA expression was analyzed with Northern blotting using CCL20, TNF-α, IL-12p35, IFN-γ, and IL-10 cDNA probes. Ethidium bromide staining was used to control equal sample loading and the integrity of RNA; **B**: Cell culture supernatants from the experiment described in panel A were collected and cytokine levels in the supernatants were determined by ELISA. The bars and error bars represent the means and standard deviations from four different blood donors. One representative experiment out of two is shown. See Figure 3 for abbreviations of bacteria.

cytokines was observed (Figure 5). The ERK MAPK inhibitor PD98059[41] had no effect on cytokine production in *B. breve* Bb99 stimulated moDCs. The NFAT inhibitor CsA had a weak inhibitory effect on Bb99-induced IFN-γ production. The production of CCL20 was enhanced in the presence of LY294002, PDTC, and CsA. Likewise, TNF-α production was enhanced in the presence of PDTC and CsA (Figure 5).

The viability of inhibitor-treated moDCs was monitored with propidium iodide (PI) staining. Treatment of moDCs with signaling inhibitors LY294002 or PDTC had a minor effect on cell viability, and approximately 70% of cells were alive at 24 h after the use of these inhibitors. P38 or NFAT inhibitors did not have effects on cell viability. In untreated control cells 86% of the moDCs were viable (data not shown).
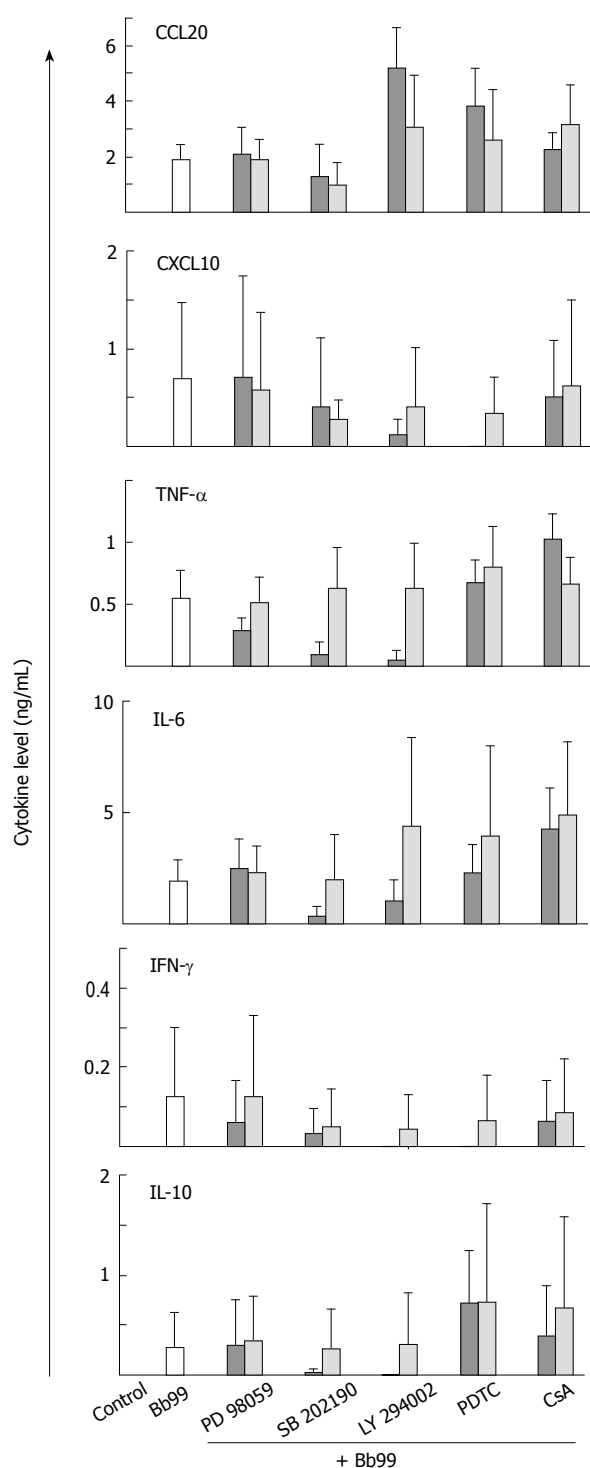
Figure 5 Pharmacological signaling inhibitors interfere with *Bifidobacterium breve* Bb99 induced cytokine production in moDCs. moDCs were left untreated or treated with different pharmacological inhibitors for 30 min prior to stimulation with Bb99 (bacteria:host cell ratio of 40:1). Cell culture supernatants from four blood donors were collected at 24 h after stimulation, and cytokine levels were determined by ELISA. The used inhibitors were: p38 MAP kinase inhibitor SB202190, PI3-kinase inhibitor LY294002, ERK MAP kinase inhibitor PD98059, NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), and NFAT inhibitor cyclosporinA (CsA). Inhibitor concentrations decrease from left to right.

Cytokine priming regulates cytokine production in bacteria stimulated moDCs

Since *B. breve* Bb99 and other studied bacteria were able to induce cytokine production in moDCs (Figure 1) we analyzed whether proinflammatory cytokines could

further contribute to probiotic bacteria-induced cytokine production and whether they would autoregulate their own production by positive or negative feedback mechanisms. MoDCs were pre-treated with IL-6, TNF-α, or IFN-γ for 16 h followed by stimulation with *B. breve* Bb99 or *S. pyogenes* for 24 h (Figure 6). IL-6 priming had a minor stimulatory effect on *S. pyogenes* induced IFN-γ and IL-10 production, whereas such an effect was not seen in *B. breve* Bb99 stimulated cells. TNF-α priming reduced IL-6, IL-10, IFN-γ, and CXCL10 production in *S. pyogenes* stimulated cells, and IL-6 and IL-10 production in *B. breve* Bb99 stimulated cells. In IFN-γ primed cells the production of IL-6, IL-10, and TNF-α was enhanced after *B. breve* Bb99 or *S. pyogenes* stimulation. In addition, the production of CCL20 and CXCL10 chemokines was enhanced in *B. breve* Bb99 stimulated cells after IFN-γ priming (Figure 6).

DISCUSSION

In the present study cytokine production profiles after probiotic bacterial stimulation were analyzed in a model system using *in vitro* cultured human moDCs. In the gastrointestinal tract bacteria are in close contact with gut epithelial cells and various types of immune cells that reside in lamina propria and the luminal side of the mucosal epithelium[42]. Mucosal DCs participate in sampling the gut microbiota by extending dendrites through the gut epithelium[15]. They have a central role in regulating both innate and adaptive immune responses during microbial infections. Therefore, it is important to study the interactions between DCs and potentially probiotic bacteria. Mucosal DCs primarily consist of myeloid DCs, which presumably are of monocyte origin[43].

We have compared the ability of two well characterized probiotics *L. rhamnosus* GG and *B. animalis* Bb12 and seven potentially probiotic bacteria to induce maturation of human moDCs. We observed that moDCs stimulated with these probiotic bacteria matured as well as moDCs stimulated with a significant human pathogen, *S. pyogenes*, as indicated by the expression of costimulatory molecules CD86 and HLA class Ⅱ. The results are well in line with previous studies which show that *S. pyogenes*, different lactobacilli, and bifidobacteria can induce DC maturation[19,33,44]. Our data shows that also *L. rhamnosus* LC705, *L. helveticus* 1129, *B. longum* 1/10, *B. breve* Bb99, *S. thermophilus* THS, *L. lactis* ARH74, and *L. mesenteroides* subsp. *cremoris* PIA2 with potential probiotic characteristics can enhance the expression of DC maturation markers. Lactobacilli seem to be able to induce DC maturation but cytokine production remains low after stimulation with *L. rhamnosus* strains LGG and LC705. Our data demonstrates that some lactobacilli are poor inducers of proinflammatory cytokines while others can stimulate cytokine production in human moDCs (Figures 1 and 2), as previously observed[44,45]. The ability of bacteria to induce moDC maturation but not cytokine production could mean that they stimulate the development of partially- or semi-mature DCs[33].
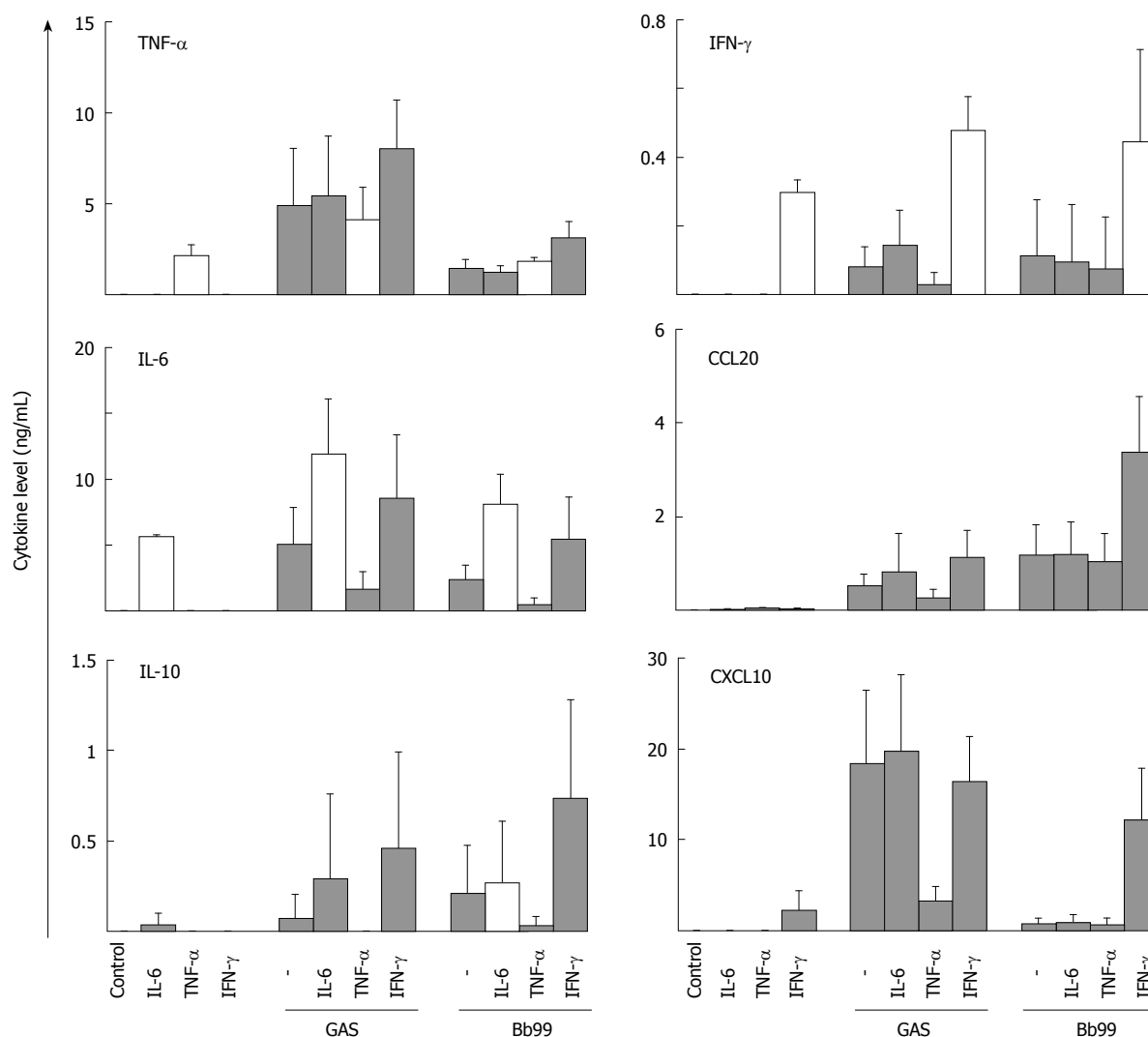
Figure 6 Pre-treatment of human moDCs with proinflammatory cytokines affects bacteria induced cytokine production. MoDCs were primed with IL-6, TNF-α, or IFN-γ for 16 h. Unprimed or cytokine primed cells were stimulated with GAS or Bb99 (bacteria:host cell ratio of 40:1). Cell culture supernatants were collected at 24 h after stimulation, and cytokine levels in supernatants were determined by ELISA. The experiment was done with cells obtained from four blood donors, and results of a representative experiment out of two are shown. The data is shown as the means, and the error bars represent standard deviations. White bars indicate cytokine priming. GAS; *Streptococcus pyogenes*; Bb99: *Bifidobacterium breve* Bb99.

This type of DCs may be involved in the induction of tolerance, as previously suggested[46].

The potentially probiotic and probiotic bacteria used in our study were previously analyzed by Kekkonen *et al*[29] in peripheral blood mononuclear cells (PBMC). In PBMC, *L. mesenteroides* subsp. *cremoris* PIA2 and *S. thermophilus* THS were potent inducers of IL-12 and IFN-γ production. However, in moDCs *S. thermophilus* THS was a potent inducer of IL-12 but not that of IFN-γ. *L. mesenteroides* subsp. *cremoris* PIA2 did not induce IL-12 or IFN-γ production in moDCs. This data demonstrates that *S. thermophilus* THS can induce a proinflammatory cytokine response in several primary cells analyzed. As shown in the present study, we are the first ones to demonstrate IFN-γ production in human moDCs in response to stimulation with potentially probiotic *B. animalis* subsp. *lactis* Bb12, *B. breve* Bb99, *L. lactis* ARH74, and *L. helveticus* 1129.

We observed that *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99 were good inducers of IL-10 production

in moDCs. *B. animalis* subsp. *lactis* Bb12 and *B. breve* Bb99 have been reported to induce IL-10 also in PBMCs[29] and other bifidobacteria in human colonic lamina propria DCs[45]. IL-10 has anti-inflammatory effects by decreasing IL-12 production and thereby also IFN-γ production which leads to the development of Th2 or Th3 type immune responses[19]. The ability of bifidobacteria to induce IL-10 production may be one factor contributing to their observed anti-inflammatory activities[45,47]. The proinflammatory cytokines produced by probiotic bacteria stimulated moDCs are suggested to induce low-grade inflammation that might protect allergy prone people from the development of allergy[48]. The ability of probiotics to induce distinct moDC cytokine production profiles in a genera or a strain-specific manner could be due to different molecules and structures expressed on the surface of these bacteria. Bacteria can also secrete active proteins or peptides that can stimulate host cells[49-51]. Recognition of these components by receptors on host cell surface can result

in the activation of diverse signaling pathways and trigger differential cytokine production.

Probiotic bacteria used in our study differentially induced moDC cytokine gene expression. *L. rhamnosus* strains LGG and LC705, and *L. mesenteroides* subsp. *cremoris* PIA2 were weak in inducing moDC cytokine responses. Bifidobacteria, *S. thermophilus* THS, *L. lactis* ARH74, and *L. helveticus* 1129 induced the production of proinflammatory cytokines TNF-α, IL-6, IL-12, and IFN-γ as well as anti-inflammatory IL-10. Pathogenic *S. pyogenes* and *S. thermophilus*, a streptococcus used in yogurt fermentation induced production of proinflammatory cytokines and chemokines TNF-α, IL-6, IL-12, CCL20, and CXCL10. IL-10 production was not observed suggesting that at least these two streptococcal species have a tendency to enhance inflammatory cytokine production. It has previously been shown that some streptococci or their components utilize TLR2 pathway to activate host cells[52,53]. Since these bacteria are good inducers of IL-12, that can not be efficiently triggered through TLR2[54], it is likely that other receptor/signaling systems apart from TLR2 are also activated. It is of interest that *L. lactis* subsp. *cremoris* ARH74 and *L. helveticus* 1129 were able to induce both strong inflammatory cytokine/chemokine and anti-inflammatory IL-10 responses.

The kinetics of bacteria-induced cytokine mRNA expression was studied with *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH7. The expression of CCL20 and TNF-α mRNAs, and CCL20, TNF-α, and IL-6 protein production was detected at early time points after stimulation with these three probiotic bacteria. Fast TNF-α and IL-6 production enhances inflammatory responses. CCL20 attracts immature DCs to the site of infection where they can be activated[55]. The fact that *S. thermophilus* THS, *B. breve* Bb99, and *L. lactis* subsp. *cremoris* ARH74 can induce efficient TNF-α, IL-6, and CCL20 production suggests that they all can trigger efficient inflammatory responses. CCL19 is a chemokine produced by activated DCs that attracts immature T cells to the local lymph nodes where their activation takes place[55]. It was of interest that none of the bacteria used in this study could induce CCL19 mRNA or protein expression. The lack of CCL19 production could indicate that the immune response triggered by probiotic bacteria may be incomplete compared to moDC responses to pathogenic bacteria, such as *S. enterica* serovar Typhimurium which is known to induce CCL19 production[56]. Th1 type cytokines IL-12 and IFN-γ were produced at later time points after stimulation with *S. pyogenes* and probiotic bacteria as shown previously with *S. pyogenes*[33]. Our results are well in line with previous experiments done with human moDCs showing that TNF-α, IL-12, and CCL20 production is induced in response to *S. pyogenes* stimulation, while a probiotic *L. rhamnosus* GG was weak in activating moDC cytokine production[33].

We used pharmacological signaling inhibitors to analyze the host cell signaling pathways regulating probiotic bacteria-induced cytokine production. Our results show that p38 MAPK, PI3 kinase, and NF-κB signaling pathways play an essential although a differential role in the ability of probiotic bacteria to induce moDC cytokine gene expression. MAPK and PI3K pathways are involved in TNF-α, IL-6, IL-10, IFN-γ, and CXCL10 production in *B. breve* Bb99 stimulated moDC. Inhibition of NF-κB and NFAT pathways led to a more limited effect on CXCL10 and IFN-γ production induced by *B. breve*. Surprisingly, the use of some inhibitors resulted in enhanced *B. breve* Bb99 induced cytokine production. Blocking one signaling pathway likely leads to stimulation of an alternative signaling pathway thus increasing cytokine production[57].

Since *B. breve* Bb99 was efficient in inducing moDC cytokine production (Figure 1), we studied the effects of different cytokines on the ability of this bacterium to induce the production of other cytokines. IFN-γ priming enhanced *B. breve* Bb99 and *S. pyogenes* stimulated production of cytokines. The effects of IFN-γ are likely due to the enhanced expression of TLRs and other signaling components, which are under the transcriptional regulation of IFNs[58]. Since IFN-γ priming increased moDC responsiveness to *B. breve* Bb99 and *S. pyogenes* it may be that identical signaling pathways are involved in cytokine production in response to stimulation with non-pathogenic *B. breve* Bb99 and pathogenic *S. pyogenes*. TNF-α priming had an opposite effect on moDC cytokine production. TNF-α is involved in moDC maturation, which decreases the capacity of moDCs to take up microbes or their components[59]. Therefore, TNF-α primed cells could be less responsive to *B. breve* stimulation. However, further studies are needed to reveal the mechanisms and co-operativity of signaling pathways involved in cytokine production in bacteria-stimulated moDCs.

In the present study carried out with human primary DCs we compared nine potentially probiotic bacteria in their abilities to induce cytokine and chemokine production in moDCs. We demonstrate that these bacteria have strain-specific effects on moDC cytokine production and they all induce moDC maturation as efficiently as pathogenic bacteria. This data is valuable for selecting new probiotic bacteria. The knowledge of unique cytokine production profiles may help in targeting specific probiotic strains for clinical applications.

COMMENTS

Background

Probiotic bacteria are widely used to relieve the symptoms of many disorders. However, the mechanisms that cause these beneficial actions are yet to be characterized. One of the factors contributing to the health promoting effects of probiotic bacteria could be their capacity to induce cytokine production that further regulates the development of innate and adaptive immune responses.

Innovations and breakthroughs

At present the cytokine production profiles of different probiotic strains, some of which have already been used in clinical trials, are poorly characterized. Also comparative data from same experimental model systems is limited. In this study we have systematically analyzed the ability of nine different potentially probiotic bacteria to induce maturation and cytokine production in human monocyte-derived dendritic cells (moDCs) in order to compare their capacities to activate innate and adaptive immune responses.

Applications

Data shows that probiotic bacteria induced the maturation of moDCs in a dose-dependent manner. They induced moDC maturation as efficiently as pathogenic bacteria but differed in a genera-specific manner in their ability to induce moDC cytokine gene expression. *S. thermophilus* induced the expression of pro-inflammatory (TNF-α, IL-12, IL-6, and CCL20) and Th1 type (IL-12 and IFN-γ) cytokines, while *B. breve* and *L. lactis* were also potent inducers of anti-inflammatory IL-10. Mitogen-activated protein kinase (MAPK) p38, phosphatidylinositol 3 (PI3) kinase, and nuclear factor-kappa B (NF-κB) signaling pathways were shown to be involved in bacteria-induced cytokine production. The cytokine profiles of probiotic bacteria can be useful in selecting new probiotic bacteria for *in vivo* trials and help to understand the mechanisms behind probiotic actions.

Peer review

This paper describes the induction of cytokines and DC maturation by different potentially probiotic bacteria. The study clearly describes the potential of probiotic bacteria to induce DC maturation, dose-dependent responses, cytokine production kinetics, and signaling pathways involved in cytokine gene expression. It's interesting.

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