

## Differential Toll-Like Receptor Recognition and Induction of Cytokine Profile by *Bifidobacterium breve* and *Lactobacillus* Strains of Probiotics<sup>▽</sup>

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**The use of probiotics as a food supplement has gained tremendous interest in the last few years as beneficial effects were reported in gut homeostasis and nutrient absorption but also in immunocompromised patients, supporting protection from colonization or infection with pathogenic bacteria or fungi. As a treatment approach for inflammatory bowel diseases, a suitable probiotic strain would ideally be one with a low immunogenic potential. Insight into the immunogenicities and types of T-cell responses induced by potentially probiotic strains allows a more rational selection of a particular strain. In the present study, the bacterial strains *Bifidobacterium breve* (NumRes 204), *Lactobacillus rhamnosus* (NumRes1), and *Lactobacillus casei* (DN-114 001) were compared concerning their capacity to induce inflammatory responses in terms of cytokine production by human and mouse primary immune cells. It was demonstrated that the *B. breve* strain induced lower levels of the proinflammatory cytokine gamma interferon (IFN- $\gamma$ ) than the tested *L. rhamnosus* and *L. casei* strains. Both *B. breve* and lactobacilli induced cytokines in a Toll-like receptor 9 (TLR9)-dependent manner, while the lower inflammatory profile of *B. breve* was due to inhibitory effects of TLR2. No role for TLR4, NOD2, and C-type lectin receptors was apparent. In conclusion, TLR signaling is involved in the differentiation of inflammatory responses between probiotic strains used as food supplements.**

In recent years, probiotic supplements have been suggested to provide health benefits. Subsequently, the use of specific probiotic strains as safe supplements for human consumption has been approved. Probiotic strains are classified as being live microorganisms which, when administered in sufficient amounts, confer a health benefit to the host (11). This health benefit could comprise more efficient digestion, nutrient absorption, or higher resistance to pathogenic bacteria in the gut.

Microorganisms from the genera *Lactobacillus* and *Bifidobacterium* are often considered as probiotic candidates. All these bacteria are Gram positive, (facultative) anaerobe microorganisms that are common commensals in the human gastrointestinal tract. These bacteria are currently used in probiotic dietary products, and several lines of evidence have demonstrated their beneficial effects on gut homeostasis (6, 16, 20, 38, 44). In addition, mouse studies have been conducted to investigate the immunomodulatory capacity of these potential probiotics, with similar findings (18, 23, 43, 46). These effects range from downregulation of cytokine responses in immune

cells to induction of apoptosis in T cells and vaccine-improving properties (4, 7, 8, 10, 13).

Previous studies indicate that the ability of probiotics to induce the secretion of various cytokines is mediated to a large extent by cell wall components (13, 25, 40). Cell wall components elicit these responses through recognition by pattern recognition receptors (PRRs), germ line-encoded receptors expressed on innate immune cells that are specialized to bind these bacterial substances. Examples of these receptors, either membrane bound or localized intracellularly, are the Toll-like receptors (TLRs), C-type lectin receptors (CLRs) (such as dectin 1, mannose receptor, or DC-SIGN), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (such as the peptidoglycan receptor NOD2). For the triggering of intracellular receptors, the process of phagocytosis is required, whereas membrane-bound receptors detect their ligand on the cell surface and in some cases facilitate phagocytosis (2). Subsequently, downstream intracellular signaling from these receptors results in the modulation of cytokine responses (30).

The PRRs that are responsible for the recognition of these strains, resulting in induction of cytokine responses and activation of the immune system, remain elusive. Therefore, in the present study the capacity of the bacterial strains *Bifidobacterium breve* (NumRes204), *Lactobacillus rhamnosus* (NumRes1), *Lactobacillus casei* (DN-114 001) to induce immune responses in

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peripheral blood mononuclear cells (PBMCs) has been examined for both healthy volunteers and NOD2-deficient Crohn's disease (CD) patients. Furthermore, the role of several PRRs of the innate immune system that could mediate these immune responses, including TLRs, CLRs, and NLRs, has been investigated in both human and mouse cells.

## MATERIALS AND METHODS

**Subjects.** Healthy volunteers and Crohn's disease patients were recruited at the Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands. The study was approved by the Ethical Committee of the Radboud University, and the volunteers gave informed consent. At the time of donation, Crohn's disease patients homozygous for the NOD2 frameshift mutation were in a quiescent phase, i.e., a prolonged period of at least 3 months of mild disease without relapses or exacerbations in the absence of immunomodulatory therapy. Also, they had received no immunomodulatory or anti-inflammatory medications for the last 3 months.

**Mice.** Wild-type C57BL/6 mice were obtained from Charles River WIGA (Sulzfeld, Germany) GmbH. TLR2 knockout mice and TLR4 knockout mice, with a C57BL/6 background, were kindly provided by S. Akira (Osaka University, Osaka, Japan) (42). All animal experiments were approved by the Animal Experimental Committee of Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands, and were performed in accordance with institutional and national guidelines.

**Reagents.** Blocking monoclonal antibodies (Abs) of the innate immune receptors TLR2 (clone T2.5) and DC-SIGN (clone AZND1), including the IgG1 isotype control, were purchased from eBioscience, Coulter Beckman, and R&D Systems, respectively, and were all used in a concentration of 10  $\mu$ g/ml. Blocking reagents of the receptors TLR4 (*Bartonella quintana* lipopolysaccharide, [31], 1  $\mu$ g/ml), TLR9 (CpG ODN TTAGGG, 25  $\mu$ g/ml; Invivogen), mannose receptor (mannan, 100  $\mu$ g/ml; Sigma), and dectin 1 (laminarin, 100  $\mu$ g/ml; Sigma), and of phagocytosis (cytochalasin B, 1  $\mu$ g/ml; Biomol International) were used.

**Bacterial fermentation and enumeration.** Two *Lactobacillus* strains (NumRes1 and DN-114 001) and a *Bifidobacterium breve* strain (NumRes204) were grown at 37°C in a 400-ml reactor containing MRS supplemented with 0.5 g/liter L-cysteine for *Bifidobacteria*. The pH was maintained at 6.5 by addition of NaOH. To ensure anaerobic conditions, the headspace was flushed with N<sub>2</sub> or a gas mixture consisting of 5% H<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> for *Bifidobacteria*. Bacteria were harvested in the early stationary phase, washed in phosphate-buffered saline (PBS), and stored with 20% (wt/vol) glycerol in aliquots at -80°C. Cell counts were determined by plating serial dilutions (CFU) and fluorescent microscopy by staining with 4',6-diamidino-2-phenylindole (DAPI).

**PBMC stimulation experiments.** Venous blood was drawn from the cubital vein of healthy volunteers into 10-ml EDTA tubes (Monoject). The mononuclear cell fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque medium (Pharmacia Biotech, Pennsylvania). Cells were washed twice in saline and suspended in culture medium (Dulbecco's modified Eagle medium [DMEM], Invitrogen, CA) supplemented with gentamicin (50  $\mu$ g/ml), L-glutamine (10 mM), and pyruvate (10 mM). Cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 5  $\times$  10<sup>6</sup> cells/ml. A total of 5  $\times$  10<sup>5</sup> mononuclear cells (MNCs) in a 100- $\mu$ l volume were added to round-bottom 96-well plates (Greiner) and incubated with either 100  $\mu$ l of culture medium (negative control) or *B. breve*, *L. rhamnosus*, or *L. casei* (10<sup>7</sup> microorganisms/well for each) at a ratio of 20:1 bacterial cells/MNCs for 24 h, 48 h, or 7 days, alone or in combination with one of the above-mentioned receptor blockers or inhibitors.

**Splenocyte stimulation experiments.** Wild-type, TLR2KO, and TLR4KO C57BL/6 mice were sacrificed, and spleens were collected. From each total spleen, a cell suspension was made in culture medium (Iscove's modified Dulbecco's medium [IMDM]; Invitrogen, California) supplemented with gentamicin (10  $\mu$ g/ml), ultraglutamine (1 mM), and 9% fetal calf serum (FCS). A total of 2  $\times$  10<sup>8</sup> splenocytes/100- $\mu$ l volume were added to round-bottom 96-well plates (Greiner) and incubated with either 100  $\mu$ l of culture medium (negative control) or the various bacterial strains: *B. breve*, *L. rhamnosus*, and *L. casei*, all in a concentration of 4  $\times$  10<sup>6</sup> microorganisms/well (bacterial cell/MNC ratio = 2:1). After 48 h of incubation at 37°C, supernatants were collected and gamma interferon (IFN- $\gamma$ ) detection was performed. A limited amount of cell death was observed (but with total viability higher than 90%), and no significant differences were seen in cell death between the different bacterial strains.

**Transfection and stimulation of TLR2-expressing HEK293 cells.** Stable TLR2-expressing HEK293 cells (Invivogen, California), containing an NF- $\kappa$ B

luciferase reporter construct (pNIFTY; Invitrogen, California), were maintained in DMEM plus 10% FCS and the appropriate antibiotics. Cells were seeded in 96-well plates and incubated overnight with bacteria at different ratios of cells to bacteria. Luciferase activity present in cell lysates was determined by detecting chemiluminescence after incubation with BriteLite (Perkin Elmer, Massachusetts).

**Generation and stimulation of mouse BMDCs.** Femurs and tibiae of female 6- to 12-week-old C57BL/6 mice were removed and purified from surrounding muscle tissue. The protocol used for cell isolation and bone marrow-derived dendritic cell (BMDC) culture with only granulocyte-macrophage colony-stimulating factor (GM-CSF) was adapted from previous publications (21, 22). Modifications were the following. At day zero, cells were plated in petri dishes (10 cm; Greiner) at 4  $\times$  10<sup>6</sup> cells per well. The medium contained 20 ng/ml of mouse GM-CSF (Peprotech). At day three, 4 ml of medium was added containing 37.2 ng/ml GM-CSF. At day 6, again 1 ml of medium was added, now containing 158 ng/ml GM-CSF. Immature dendritic cells (DCs) were harvested at day seven and used in the cytokine assay. A cell suspension was made (1  $\times$  10<sup>6</sup> cells/ml), and the cells were plated in 96-well plates. To the BMDCs either probiotics (bacterial cell/BMDC ratio = 1:1) or Pam3Cys (4  $\mu$ g/ml) was added. DCs were incubated with these stimuli for either 24 h or 48 h depending on the cytokine to be measured.

**Cytokine measurements.** Cytokine production capacity was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. ELISA kits were purchased from R&D Systems (Minnesota) for tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), and IL-17 and from Sanquin (Amsterdam, Netherlands) for IL-6, IL-10, and IFN- $\gamma$ . Mouse IFN- $\gamma$  cytokine production capacity was measured by ELISA according to the protocol reported previously (45). In brief, MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 1  $\mu$ g/ml purified rat anti-mouse IFN- $\gamma$  (capture Ab; BD Pharmingen) overnight at 4°C in 0.1 M carbonate buffer. Plates were washed with 0.05% Tween-PBS and blocked with 1% bovine serum albumin (BSA)-1% FCS in PBS for 1 h at room temperature (RT). Plates were washed, and the samples and standards were incubated for 1 h at 37°C. Subsequently, wells were washed and incubated with 0.5  $\mu$ g/ml biotin-conjugated rat anti-mouse IFN- $\gamma$  (clone XMG1.2; Biolegend) in 0.1% BSA-0.1% FCS in PBS for 1 h at RT, followed by streptavidin-horseradish peroxidase (Invitrogen) and tetramethylbenzidine substrate (SFRI Laboratories, Berganton, France). The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>, and adsorption was measured at 450 nm using a Bio-Rad multiplate reader (Bio-Rad Laboratories, Veenendaal, Netherlands).

**Statistics.** Differences in cytokine production capacity between groups were analyzed using the Mann-Whitney U test. Differences were considered statistically significant at *P* values of <0.05.

## RESULTS

**Cytokine production capacity of PBMCs induced by potentially probiotic strains.** To study the immunostimulatory capacities of the bacterial strains *B. breve*, *L. rhamnosus*, and *L. casei*, PBMCs were stimulated with bacterial cells at a ratio of 20:1, and cytokines were measured at different time points. Important differences were observed between induction of cytokine production by the bacterial strains. Most importantly, while all monocyte-derived cytokines can be induced by all probiotic strains studied, only lactobacilli, but not *B. breve*, stimulated production of IFN- $\gamma$  (Fig. 1).

**Role of membrane-bound receptors.** To study the role of particular PRRs in the cytokine responses induced by the bacterial strains, several PRR-specific blocking agents were applied to the PBMCs before administration of the bacterial strains. Surprisingly, blocking of TLR2 resulted in increased production of IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  when cells were stimulated with *B. breve* (Fig. 2). However, no differences were observed when TLR2 was blocked, either in production of IL-6, IL-10, and IL-17 (data not shown) or after stimulation with *L. rhamnosus* or *L. casei* (Fig. 2). Stimulation of mouse BMDCs with Pam3Cys or either *Lactobacillus* strain also demonstrated a minor role for TLR2 in recognition of these strains

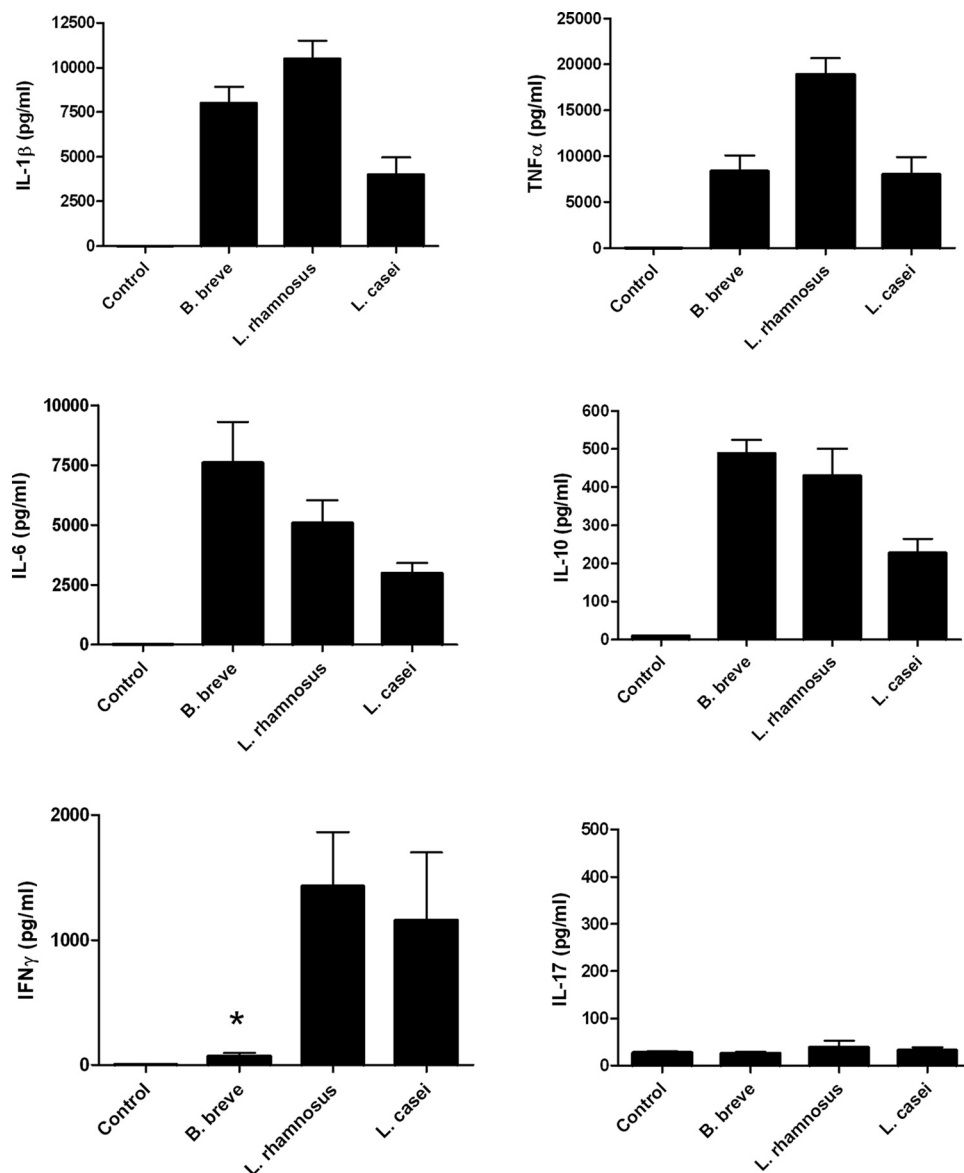


FIG. 1. Cytokine production capacity of PBMCs collected from healthy volunteers and stimulated with the bacterial strains for 24 h (IL-1 $\beta$ , TNF- $\alpha$ , and IL-6), 48 h (IL-10 and IFN- $\gamma$ ), or 7 days (IL-17). Values are means  $\pm$  SEM;  $n = 4$ ; \*,  $P < 0.05$  compared to results for stimulation with *L. rhamnosus* or *L. casei*.

(Fig. 3A). Accordingly, additional experiments with TLR2-transfected HEK293 cells revealed that *B. breve* but not *L. rhamnosus* or *L. casei* induces TLR2 signaling (Fig. 3B). Blocking of TLR4 had no effect on cytokine production. These experiments were also performed with mouse splenocytes, either wild type, TLR2 knockout (KO), or TLR4 KO. Consistent with the human data, IFN- $\gamma$  responses in TLR2 KO cells but not in TLR4 KO cells were different from those in wild-type cells after stimulation with *B. breve* (Fig. 3C). Compared to the human data, the TLR2 KO splenocytes also exhibited a further increase in the production of IFN- $\gamma$  relative to results with wild-type splenocytes after stimulation with *L. rhamnosus* and *L. casei*, whereas the TLR4 KO cells did not. Blocking the C-type lectin receptors dectin 1, mannose receptor, and DC-

SIGN did not significantly influence probiotic-induced cytokine production (data not shown).

**Role of intracellular receptors.** Microbial recognition at the level of the cell membrane is complemented by pattern recognition in the intracellular compartment by TLRs (e.g., TLR9) or NLRs (e.g., NOD2). Therefore, the role of phagocytosis was studied by using the phagocytosis inhibitor cytochalasin B. Cytochalasin B decreased cytokine responses for virtually all cytokines, suggesting an important role for intracellular recognition receptors (Fig. 4). Therefore, the role of intracellular PRRs, such as TLR9 and NOD2, in recognition of these bacterial strains was studied. Blocking of the TLR9 receptor resulted in a profound decrease in IL-10 and IFN- $\gamma$  responses after stimulation of cells with either bacterial strain (Fig. 5).

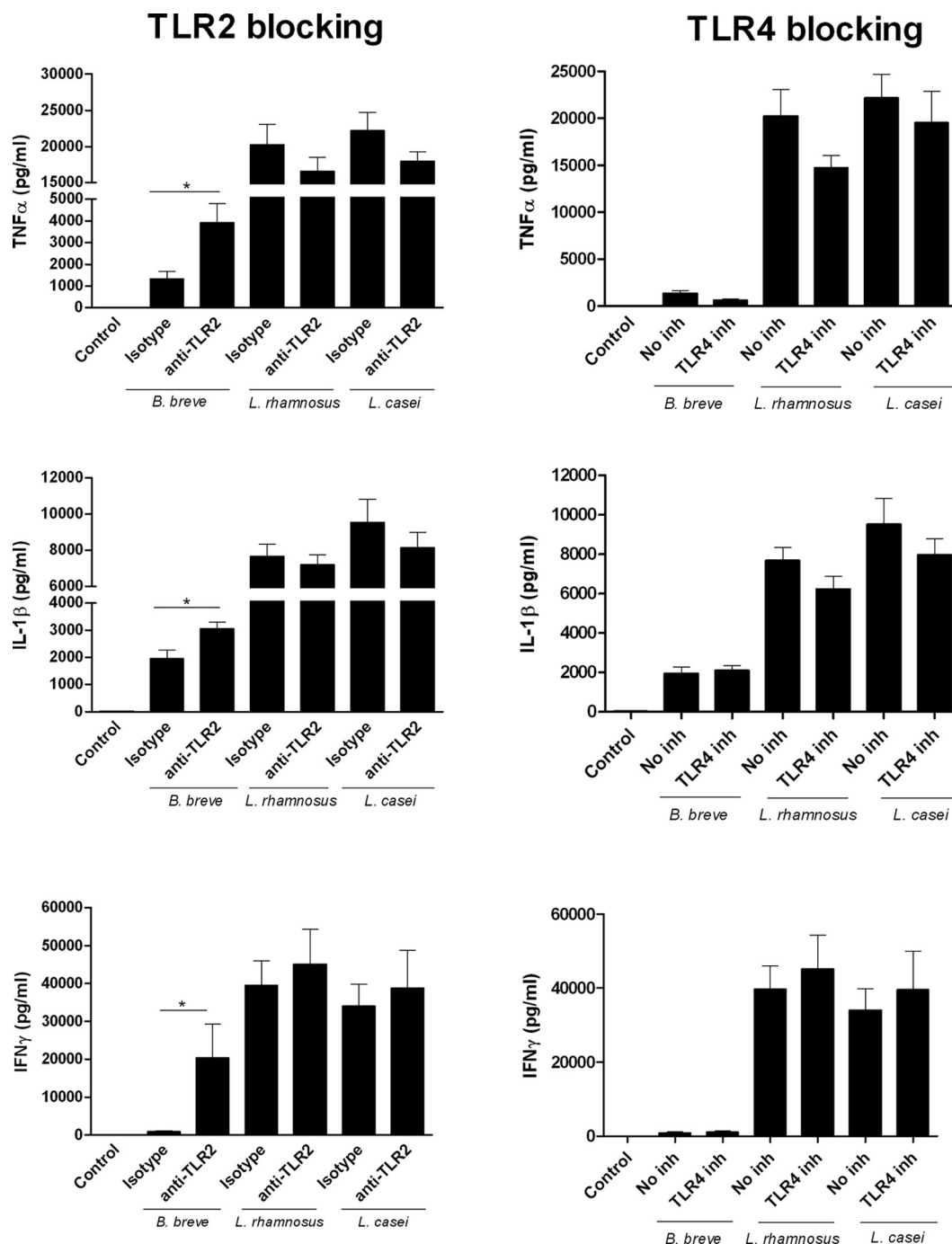


FIG. 2. Cytokine production capacity of TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$  by PBMCs obtained from healthy volunteers and stimulated with the bacterial strains together with a TLR2 blocking antibody or a TLR4 inhibitor (inh). Values are means  $\pm$  SEM;  $n = 6$ ; \*,  $P < 0.05$ .

For the other cytokines measured, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 concentrations revealed no differences between the conditions (data not shown). To assess the role of the intracellular PRR NOD2, cells from healthy subjects with functional NOD2 were compared with cells obtained from Crohn's disease (CD) patients that are homozygous for the 1007fsinsC frameshift mutation in NOD2, leading to a loss of function of the protein. These analyses revealed no apparent differences between the

two groups after stimulation of these cells with the bacterial strains (Fig. 6).

## DISCUSSION

Nowadays, dietary products are widely supplemented with probiotics, i.e., live microorganisms that may have the ability to provide a health benefit to the host. Desirable features of a

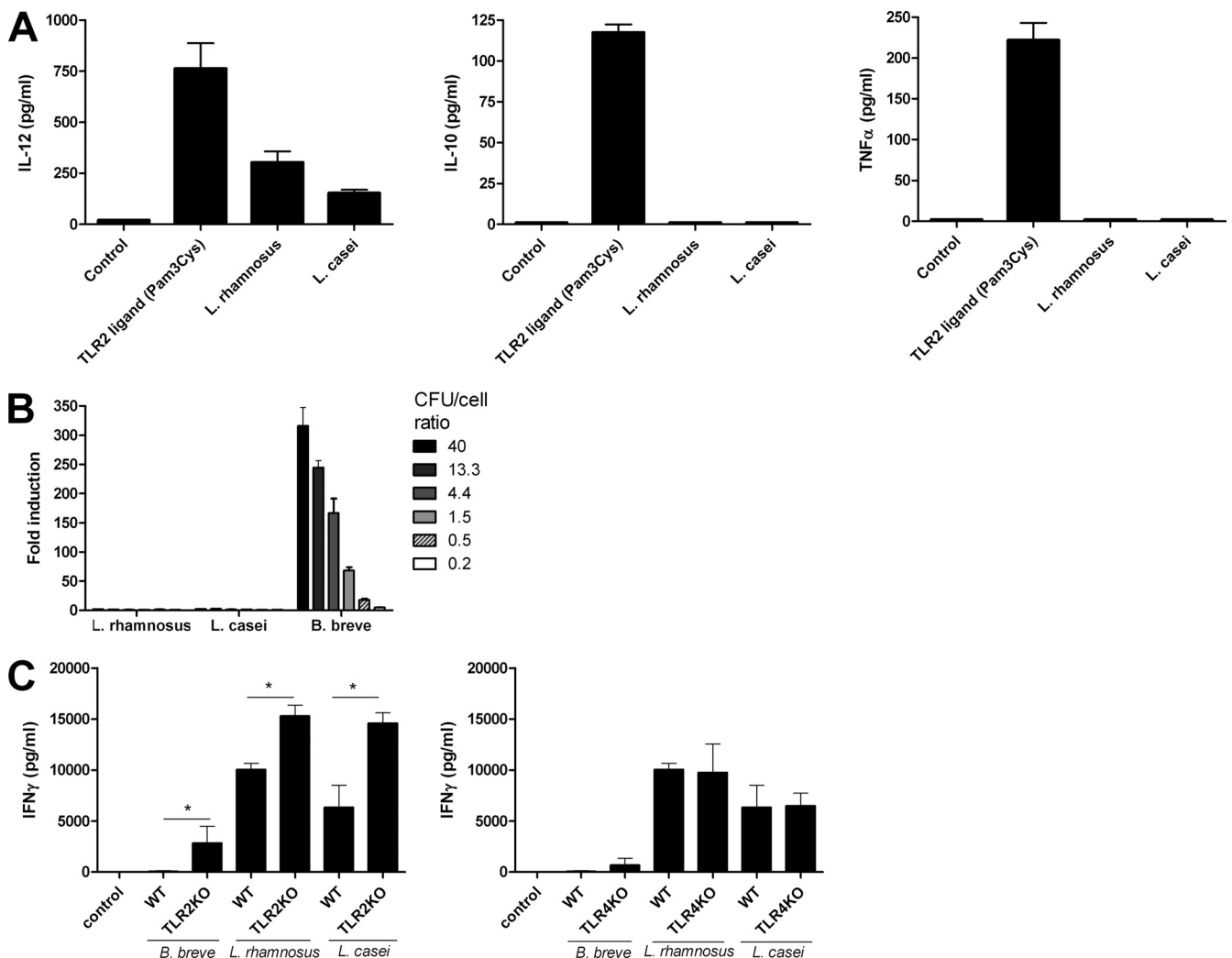


FIG. 3. (A) Production of IL-12, IL-10, and TNF- $\alpha$  after stimulation of mouse bone marrow-derived dendritic cells with Pam3Cys, *L. rhamnosus*, or *L. casei* for 24 or 48 h. Values are means  $\pm$  SEM;  $n = 4$ . (B) NF- $\kappa$ B activity after overnight stimulation of TLR2-transfected HEK293 cells with different CFU/cell ratios of *B. breve*, *L. rhamnosus*, or *L. casei*. Fold induction was calculated as the ratio of experimental activity to control activity. Values are means  $\pm$  SD;  $n = 4$ . (C) Cytokine production capacity of IFN- $\gamma$  by splenocytes obtained from wild-type (WT), TLR2KO, and TLR4 KO mice stimulated with the bacterial strains. Values are means  $\pm$  SEM;  $n = 6$ ; \*,  $P < 0.05$ .

probiotic strain are protective colonization of the intestinal tract and increasing efficiency of digestion and nutrient absorption but also a low capacity to cause immune responses to deviate toward Th1 and/or Th17 immune responses, which are associated with intestinal pathology (32, 37, 39). Hence, the potential of probiotics as a therapeutic moiety in diseases involving the gastrointestinal tract (e.g., infections, inflammatory bowel disease, and colon cancer) has been suggested (12, 33, 47). The rational use of probiotics in health and disease, however, needs a detailed understanding of the recognition pathways that can activate host defense by probiotics.

The potential of probiotics to modulate immune responses may represent an important factor for their therapeutic application. In fact, it is known that the release of TNF- $\alpha$  by inflamed Crohn's disease mucosa can be significantly reduced by coculture with *L. casei* DN-114 001 (5). More specifically, *L. casei* can counteract the proinflammatory effects of *Escherichia coli* on Crohn's disease-inflamed mucosa by specific downregu-

lation of key proinflammatory mediators (19). In the present study, we compared the inflammatory properties of three different bacterial strains, *B. breve* NumRes204, *L. rhamnosus* NumRes1, and *L. casei* DN-114 001, which belong to genera that are all widely used strains in dietary products, in their capacity to evoke and thereby skew immune responses toward distinct T-helper-mediated responses (i.e., immune deviation). Compared with lactobacilli, *B. breve* was less capable of inducing IFN- $\gamma$  responses in human primary immune cells. The differential stimulation of cytokine production by *B. breve* and lactobacilli was mediated by differences in recognition by PRRs with either stimulatory or inhibitory effects on cytokine responses.

Specific blocking of particular PRRs enabled us to elucidate what receptors are involved in the activation of cytokine production. The first important observation was that cytokine induction by both *B. breve* and the lactobacilli is strongly dependent on TLR9. Blocking of TLR9 resulted in



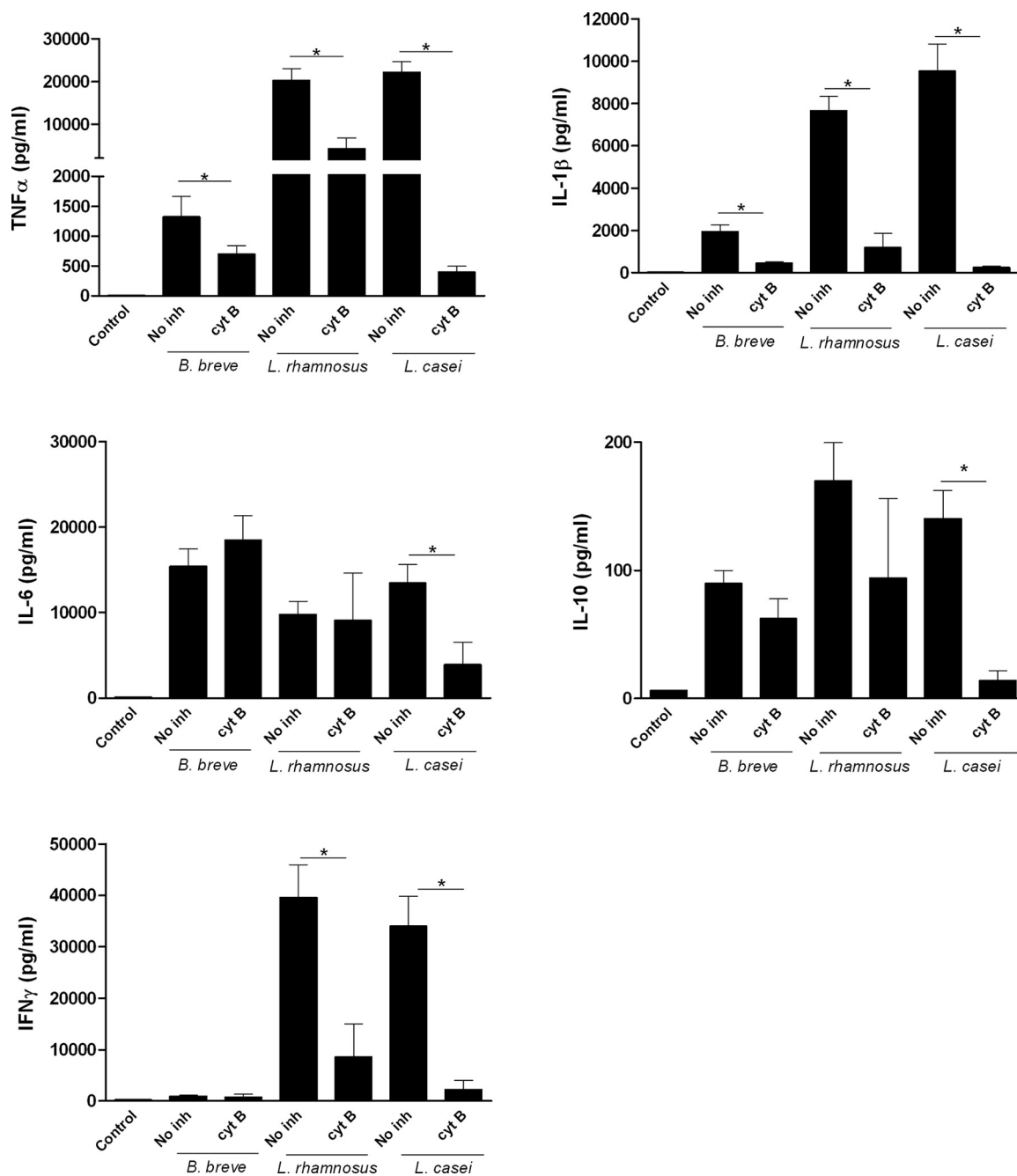


FIG. 4. Cytokine production capacity for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, and IFN- $\gamma$  by PBMCs obtained from healthy volunteers and stimulated with the bacterial strains in the absence or presence of cytochalasin B. Values are means  $\pm$  SEM;  $n = 6$ ; \*,  $P < 0.05$ .

severely decreased production of IL-10 and IFN- $\gamma$ , indicating that these bacterial strains contain immune-stimulatory double-stranded DNA (dsDNA) containing unmethylated CpG sequences. In contrast, no differences were observed when the action of other PRRs, such as TLR4, dectin 1, mannose receptor, DC-SIGN, and NOD2, was inhibited, indicating that these receptors have a minor role in recognition of bacterial strains. The role of TLR9 in recognition of bacterial (3, 14) and fungal (26, 34) DNA is well known; however, its role in the induction of innate immune re-

sponses by probiotics in primary human cells had not been demonstrated until now.

Recognition of *B. breve* by TLR2 had an effect opposite to that with TLR9. Blocking of TLR2 with a specific antibody stimulated cytokine responses induced by *B. breve*, including TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ . Interestingly, no role for TLR2 could be demonstrated in *L. rhamnosus*- and *L. casei*-induced cytokine responses, indicating either that these bacteria lack TLR2 ligands in their cell wall or, perhaps more likely, that potential TLR2 ligands are masked. Of note, bone marrow-

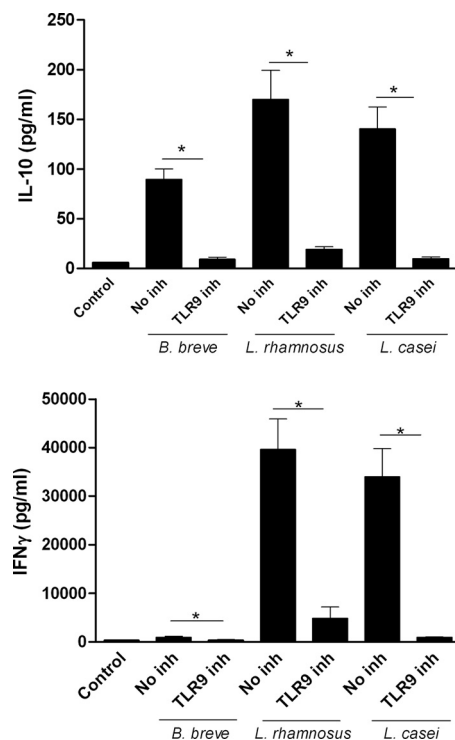


FIG. 5. Cytokine production capacity for IL-10 and IFN- $\gamma$  by PBMCs obtained from healthy volunteers and stimulated with the bacterial strains for 48 h together with a TLR9 blocking agent. Values are means  $\pm$  SEM;  $n = 6$ ; \*,  $P < 0.05$ .

derived murine dendritic cells, which are highly susceptible to TLR2 agonists, also failed to elicit noteworthy cytokine responses after exposure to these bacterial strains. In addition, data from TLR2-overexpressing human embryonic kidney (HEK293) cells containing an NF- $\kappa$ B reporter construct showed NF- $\kappa$ B induction upon incubation with *B. breve* whereas *L. rhamnosus* and *L. casei* were unable to do so. To complement and support the human data, additional experiments were performed with mouse splenocytes that were either wild type or deficient in TLR2 or TLR4. In these experiments, IFN- $\gamma$  production was increased in TLR2 KO cells but was similar in TLR4 KO cells to that in the wild type, which is consistent with the human data. These findings suggest that TLR2 induces an intracellular inhibitory signal to downstream pathways that elicit cytokine production, such as the TLR9 pathway. Indeed, this anti-inflammatory role of TLR2 has been demonstrated before, both *in vitro* and *in vivo* (1, 9, 35, 36). Moreover, TLR2 has also been implicated in the induction of regulatory T-cell responses, further emphasizing the immunosuppressive potential of TLR2 signaling (27, 28, 41). Also, a *Bifidobacterium* strain has been shown to be able to induce T-regulatory cells (23). However, it remains to be elucidated whether TLR2 is involved in this process, although previously an immunoregulatory role of TLR2 in recognition of probiotic strains was indeed described (15, 17, 48).

When considering the potential therapeutic applications of these probiotic strains, one could draw useful information from the *in vitro* features of these strains. The data presented here suggest that *B. breve* may be more suitable than Lactobacilli in

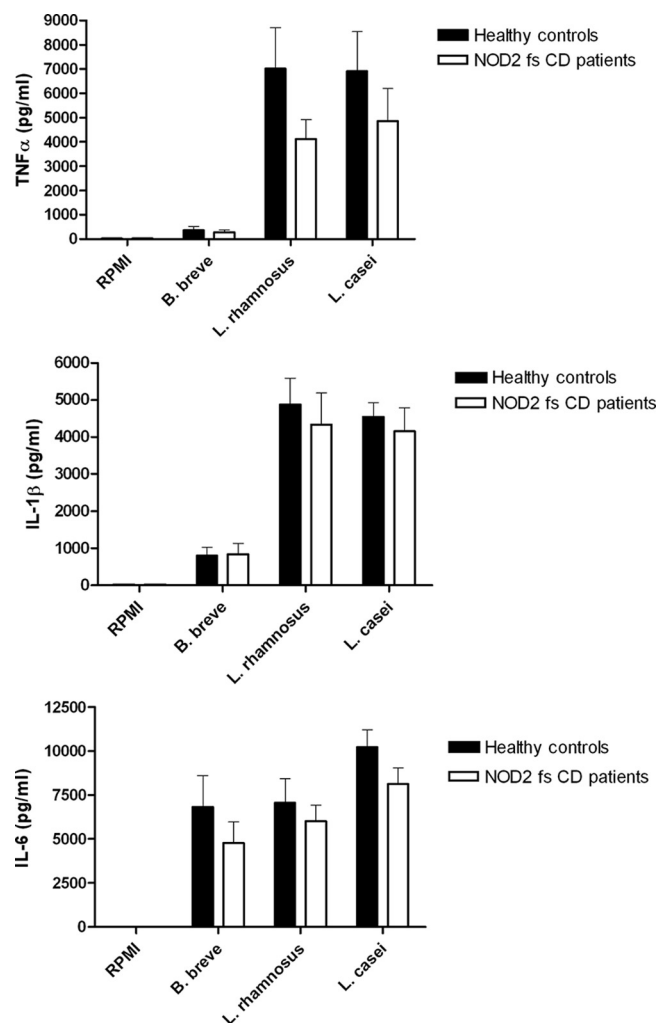


FIG. 6. Cytokine production capacity for TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 by PBMCs obtained from healthy volunteers or CD patients homozygous for the frameshift (fs) mutation in NOD2 (1007fsinsC) and stimulated with the bacterial strains for 24 h. Values are means  $\pm$  SEM;  $n = 4$ .

the context of inflammatory bowel diseases (and especially Crohn's disease), based on its lower inflammatory potential. Th1 responses, as reflected by IFN- $\gamma$  production, are poorly induced by *B. breve*, while they are known to be crucial for the pathogenesis of Crohn's disease (24, 29). Beneficial effects of the inhibition of IFN- $\gamma$  production by *B. breve*, compared to, e.g., anti-TNF- $\alpha$  treatment, may result in a tolerant state with decreased Th1 immune responses in the gut, whereas other (e.g., IL-1 $\beta$ - and TNF- $\alpha$ -driven) immune pathways remain intact, thereby sustaining immunocompetence of the host. However, further *in vivo* studies need to be performed to confirm these findings and to assess the effect on relevant animal models, such as experimental colitis.

In conclusion, the observed differences in the capacity to induce cytokine secretion in human primary immune cells between the *Bifidobacterium* and *Lactobacillus* strains is most likely due to the differential recognition by TLRs. TLR9 mediates proinflammatory signals induced by *B. breve*, *L. rham-*

*nosus*, and *L. casei*. In contrast, TLR2 exerts inhibitory effects upon recognition of *B. breve* but not lactobacilli. It is to be hoped that these data would contribute to the decision for a deliberate choice on what probiotic to use in a given application.

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