Antigen-Presenting Cells Exposed to *Lactobacillus acidophilus* NCFM, *Bifidobacterium bifidum* BI-98, and BI-504 Reduce Regulatory T Cell Activity

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Background: The effect in vitro of six different probiotic strains including *Lactobacillus acidophilus* NCFM™, *Lactobacillus salivarius* Ls-33, *Lactobacillus paracasei subsp. paracasei* YS8866441, *Lactobacillus plantarum* Lp-115, *Bifidobacterium bifidum* BI-504 and BI-98 was studied on splenic enteroantigen-presenting cells (APC) and $CD4^+CD25^+$ T-regulatory cells (Tregs) in splenocyte-T cell proliferation assays.

Methods: Splenocytes exposed to enteroantigen +/− probiotics were used to stimulate cultured $CD4^+CD25^-$ T cells to which titrated numbers of Tregs were added. Cytokine assays were performed by use of neutralizing antibodies and ELISA.

Results: Exposure of APCs to enteroantigens and the series of probiotic strains mentioned above did not influence the stimulatory capacity of APCs on proliferative enteroantigen-specific T cells. However, exposure to *B. bifidum* BI-98, BI-504 and *L. acidophilus* NCFM™ consistently reduced the suppressive activity of Tregs. The suppressive activity was analyzed using fractionated components of the probiotics, and showed that a component of the cell wall is responsible for the decreased Treg activity in the system. The probiotic-induced suppression of Treg function is not mediated by changes in APC-secretion of the inflammatory cytokines IL-6 or IL-1b.

Conclusion: We conclude that certain probiotic strains can modify APCs to cause reduced Treg activity. This effect apparently depends on a direct APC-to-Treg cell contact. The APC-mediated suppressive effect on Treg function of certain probiotic strains may constrain the anti-inflammatory activity, which is often desired from probiotic therapy. This unexpected function of certain probiotic strains should be taken into consideration when designing adjuvant therapies with these bacteria, or when probiotic strains are selected for improvement of gut-associated inflammation like IBD.

(Inflamm Bowel Dis 2010;16:390–400)

Key Words: regulatory T cells, colitis, inflammation, probiotics

Probiotics are defined as: "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Probiotics typically include different strains of lactic acid bacteria within the genera *Bifidobacterium* and *Lactobacillus*.[1,2] Beneficial immunomodulatory effects of probiotics are both indirect by induction of immunoregulatory cytokines in antigen-presenting host cells (APCs) as well as direct by competing for space with other commensal bacteria and their capacity to secrete IL-10-like and other immunoregulatory molecules. Thus, probiotics can alter the immune system by increasing the intestinal antiinflammatory cytokine level and/or reduce the proinflammatory cytokines.[3–6] They have also been shown to alter the antibody production,[7] natural killer cell activity,[8,9] as well as modulating the NF-κB pathway.[10,11] In addition, probiotics have been shown to compete for adhesion sites with other enteropathogenic bacteria and to secrete antimicrobial compounds such as different cytokine-like agents.[12,13]

There are relatively few publications on clinical trials dealing with probiotics and inflammatory bowel disease (IBD) and, consequently, there is a lack of knowledge concerning the efficacy of treatments with probiotics and their mode of action. We have previously shown that probiotics (a combination of 2 *Lactobacillus* strains) added to the drinking water of mice conferred beneficial effects in mice suffering from adoptive transfer colitis, although this treatment was not curative.[14] Feeding lactobacilli has also been shown to prevent development of colitis in IL-$10^{-/-}$ mice.[6] Studies performed in IBD suggest that high doses of probiotics and combinations of different lactobacilli and bifidobacteria are more effective in decreasing inflammatory score and maintaining patients in remission than a single probiotic strain.[15]

Received for publication June 22, 2009; Accepted June 23, 2009.

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Supported by the Danish Agency for Science, Technology and Innovation.

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DOI 10.1002/ibd.21068

Published online 27 August 2009 in Wiley InterScience (www.interscience.wiley.com).

Treatment of ulcerative colitis (UC) patients with different probiotics has yielded promising results and it seems that probiotics might be reasonably effective in maintaining medically induced remission of active IBD.[16,17]

There is a general consensus that the mucosal inflammation associated with IBD is in part due to a breakdown in tolerance toward the normal bacterial flora of the gut. This might be a result of malfunction in the regulatory T cell (Treg) compartment.[18,19] In the SCID transfer model of colitis we have shown that enterobacterial-reactive $CD4^+CD25^-$ T cells can be inhibited by $CD4^+CD25^+$ Tregs.[20] $CD4^+$ T cells from the mesenteric lymph nodes (MLNs) are unable to respond when exposed to enterobacterial antigens in vitro. However, depletion of the $CD4^+CD25^+$ T cells results in a $CD4^+CD25^-$ T cell population that proliferates extensively when exposed to enteroantigen. Several studies have demonstrated that $CD4^+CD25^+$ Tregs are able to prevent or even cure mild experimental colitis when they are adoptively transferred into immune-deficient mice.[21–23]

That probiotics might influence the Treg compartment has been suggested more recently as dendritic cells, when exposed to probiotics, seem to favor the development of Tregs.[24,25] This function of probiotics is probably mediated by an increase in the production of IL-10.[26,27] Also, administration of a mixture of 8 probiotic strains (VSL#3) to patients with ileal pouch–anal anastomosis has been shown to result in local expansion of Tregs that correlated with reduced inflammation.[28] It is, however, not as simple, since different probiotic species seem to differ in their ability to induce Tregs[29] and modulation in cytokine profiles.[26,30,31] Different isolates of *Lactobacillus acidophilus* have been demonstrated to display different effects.[32] It is thus very important to characterize each probiotic strain carefully before introducing them into clinical trials. In this article we characterize the in vitro effect of 6 different probiotic strains on the enteroantigen specific proliferation of $CD4^+CD25^-$ T cells with a colitogenic potential in the SCID transfer model as well as on $CD4^+CD25^+$ Tregs capable of preventing development of colitis.

MATERIALS AND METHODS

Mice

Female wildtype BALB/c were purchased from Taconic Farms (Lille Skensved, Denmark). The mice were allowed to rest for at least 1 week before entering experiments, at which time they were 6–8 weeks old. All animals were housed under controlled microbial environment conditions, which included testing of sentinels for unwanted infections according to the Federation of European Laboratory Animal Science Association standards; no such infections were detected.

Isolation of $CD4^+$ T cell Subsets

$CD4^+$ T cells were positively selected from mesenteric lymph node single cell suspensions using a mouse anti-CD4 mAb coated Dynabead and detach-a-bead system (Dynal, Norway) according to the manufacturer's description. The $CD4^+$ T cells (>98% pure assessed by flow cytometry) were then separated into $CD25^+$ and $CD25^-$ T cell populations by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) using PE-labeled anti-CD25 mAb followed by addition of anti-PE microbeads, according to the manufacturer's description.

Preparation of Fecal Extract

Extract was prepared by removing the colon and cecum from the relevant mice and placing the content in phosphate-buffered saline (PBS). This was sonicated 3 times 30 seconds on ice, followed by centrifugation at 10,000*g* for 10 minutes to remove insoluble material. The supernatant was collected, sterile-filtered, and stored at −80°C. The protein concentration in the supernatants was typically 1–1.5 mg/mL as determined by the BCA method.

Preparation of Probiotics

The following probiotic strains were included in the study: *L. acidophilus* NCFM ATCC 700396 (Danisco, Cultures Division, Paris, France), *L. salivarius* Ls-33 ATCC SD5208 (Danisco), *L. paracasei* subsp. *paracasei* YS8866441, *L. plantarum* Lp-115 ATCC SD5209 (Danisco), and the 2 human isolates *Bifidobacterium bifidum* BI-504 DSM 19158 (Bioneer, Hørsholm, Denmark), and *B. bifidum* BI-98 DSM 19157 (Bioneer). The strains were grown anaerobically in MRS broth (Oxoid, Nepean, Canada) at 37°C to stationary phase. For bifidobacteria, 2.8 mM L-cysteine and 20 mM DL-threonine were added to the growth medium. Cells were harvested, washed twice in PBS, and pelleted by centrifugation before freeze-drying. To determine the amount of bacteria, tubes were weighed empty and with freeze-dried bacteria.

Fractionation of Probiotics

A protoplast fraction consisting of cytoplasm and cell membrane were prepared as follows: Protoplasts were prepared according to a previous method,[33] except that lactose was used instead of sucrose as a stabilizing agent. Bacterial cells were inspected by light microscopy to ensure protoplast formation. Protoplasts were resuspended in H_2O and freeze-dried before use. Cell wall fragments and cytoplasm fractions were prepared as described,[34] except that cells were fragmented using a Fastprep cell disrupter (BIO101, La Jolla, CA) and 107 μm glass beads (Sigma-Aldrich, St. Louis, MO). Total DNA was prepared as described[35] and plasmid DNA was prepared as described.[36]

Preparation and Pulse of Splenocytes

Spleen cells from BALB/c mice were used as APCs. The splenocytes were cultured in 24-well plates at 8×10^6 cells/well in a final volume of 2 mL and 200 μg/mL of extract protein was added. After 4 hours probiotics were added in the indicated concentrations and the culture was incubated for further 20 hours at 37°C. To remove the probiotics from the cells the splenocytes were centrifuged twice in medium through fetal calf serum. After this the splenocytes were washed again in medium and irradiated (2000 rad). In selected experiments, cytokine determinations (IL-1β, IL-6, or IL-10) in the culture supernatants were performed by enzyme-linked immunosorbent assay (ELISA) (see below).

Proliferation Assay

Splenocytes (1.0×10^5) and $CD4^+CD25^-$ T cells (1.0×10^5) were added to each well of a 96-well round-bottom culture plate. In some experiments $CD4^+CD25^+$ Tregs were also added in different numbers as indicated in the individual experiments. After 4 days of culture proliferation was measured by adding 0.5 Ci ^{3}[H]-thymidine to each well, incubating for a further 18 hours and harvesting the cells to count the incorporated thymidine. In selected experiments the culture supernatants were harvested for cytokine measurements by ELISA (see below).

ELISA

Murine ELISA kits (IL-1β, IL-6, or IL-10) were used as recommended by the supplier (R&D Systems, Minneapolis, MN). The conditioned media from each incubation were diluted to reach the linear range for each ELISA assay. ELISA was made immediately after removal from the wells, or from media frozen at −80°C until analyses.

Flow Cytometry

Cells, 1×10^6, were stained with directly labeled mAbs in PBS containing 1% bovine serum albumin (BSA) for 20 minutes in the dark at 4°C. After washing twice, cells were fixed with 1% paraformaldehyde. Samples were acquired on a FACS Calibur (Becton Dickinson, San Jose, CA), and at least 10^4 mononuclear cells were gated using a combination of forward angle and sidescatter to exclude dead cells and debris. Data were analyzed using Cell-Quest software (Becton Dickinson).

Statistics

The Wilcoxon nonparametric test was employed to compare proliferation data and a *t*-test was employed to compare cytokine data; in all cases $P < 0.05$ was considered significant.

RESULTS

Stimulatory Capacity of Splenocytes Is Unaffected by Lactic Acid Bacteria

To address the potential immunomodulating effects of the following 6 different probiotic strains: *L. acidophilus* NCFM, *L. salivarius* Ls-33, *L. paracasei* subsp. *paracasei* YS8866441, *L. plantarum* Lp-115, *B. bifidum* BI-504, and *B. bifidum* BI-98 we employed a well-established immunostimulatory proliferation assay. In this, Treg-depleted $CD4^+CD25^-$ T cells, which can induce colitis by transfer to SCID mice, were specifically stimulated by splenocytes exposed to fecal extract (Fig. 1A).[20] Five days stimulation with fecal extract-pulsed splenocytes led to extensive proliferation of the $CD4^+CD25^-$ T cells, whereas an insignificant background proliferation was generated by unpulsed splenocytes. It has previously been documented that the proliferation in this system is not caused by endotoxins or superantigens but mediated by T cells and restricted by MHC class II.[20] By exposure of splenocytes to bacteria during the period of fecal extract pulsing we wanted to see whether the individual bacterial strains influence enteroantigen-specific T cell responses by modulating the stimulatory capacity of the splenocytes. However, none of the 6 bacterial strains tested affected the T cell responses significantly, as all bacteria-treated splenocytes elicited levels of T cell proliferation similar to that obtained by splenocytes pulsed with fecal extract alone (Fig. 1B).

Some Probiotic Bacteria Reduce the Suppressor Activity of Treg

The proliferation of $CD4^+CD25^-$ T cells stimulated with fecal extract-pulsed splenocytes is tightly regulated by the $CD4^+CD25^+$ Tregs, which are depleted from the responding T cell population prior to stimulation.[20] Reintroducing the Tregs at increasing cell numbers during the stimulation clearly demonstrated their regulatory role in this enteroantigen-specific system (Fig. 2A). In fact, significantly reduced T cell proliferation was observed in presence of only a few Tregs (12,5000 Tregs corresponding to a Treg:effector T cell ratio of 1:8) and ≈50% inhibition occurred at a ratio of 1:8. Tregs alone did not proliferate when stimulated with fecal extract-pulsed splenocytes (Fig. 2A).

In view of the fact that Tregs have such a strong impact on the proliferating enteroantigen-specific $CD4^+CD25^-$ T cells, we speculated whether Treg function is influenced by fecal extract-pulsed splenocytes exposed to probiotics. To test this hypothesis we stimulated $CD4^+CD25^-$ T cells with fecal extract-pulsed splenocytes exposed to the different lactic acid bacterial strains during the process of enteroantigen-processing. Activation of $CD4^+CD25^-$ T cells was carried out in the presence of

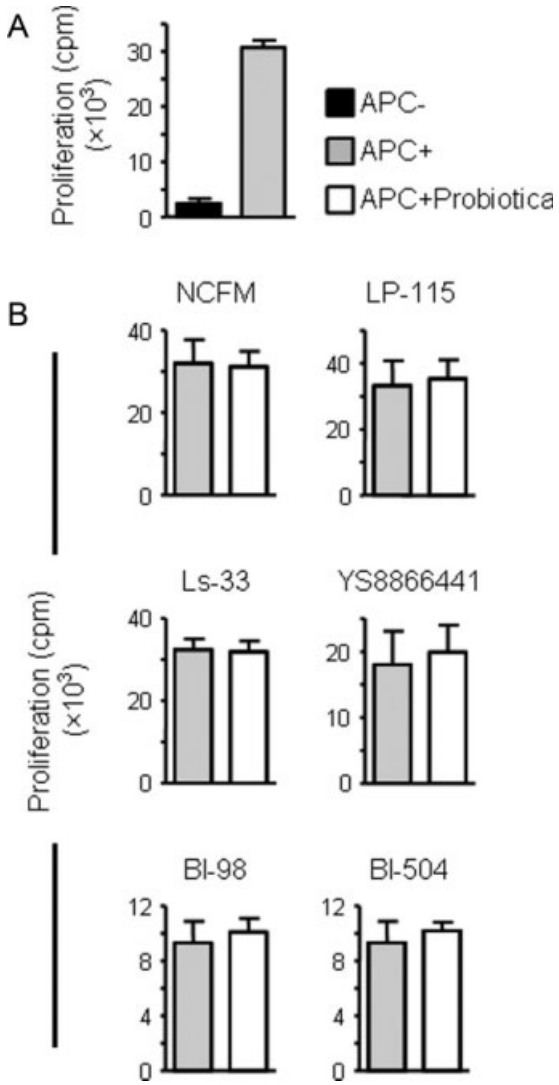

FIGURE 1. Probiotics do not alter the enteroantigen-specific stimulatory capacity of splenocytes. (A) Splenocytes were pulsed for 24 hours with fecal extract (APC+) or unpulsed (APC−) and cocultured with mesenteric lymph node $CD4^+CD25^-$ T cells for 5 days. (B) Splenocytes were pulsed for 24 hours with fecal extract alone (APC+) or in combination with probiotics (100 μg/mL) as indicated followed by coculture for 5 days with $CD4^+CD25^-$ T cells. Proliferation was measured by ^{3}[H]-thymidine incorporation. Data represent mean of quadruplicates and 1 representative of 5 experiments is shown. Error bars: SD.

increasing numbers of Tregs (Fig. 2B). Proliferation of $CD4^+CD25^-$ T cells stimulated with fecal extract-pulsed splenocytes not treated with probiotics showed the decline in proliferation, also shown in Figure 2A, correlating well with the increasing numbers of Tregs added. However, exposure of splenocytes to *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 resulted in a reduction in Treg activity being most pronounced at Treg:effector ratios of 1:8 and 1:4. In contrast, splenocytes exposed to *L. salivarius* Ls-33, *L. paracasei* YS8866441, or *L. plantarum* Lp-115 only caused insignificant variations in T cell proliferation when compared to splenocytes not exposed to probiotics.

The observed inhibitory effect on Treg activity induced by splenocytes exposed to the probiotic strains *L. acidophilus* NCFM, *B. bifidum* BI-98, and *B. bifidum* BI-504 is summarized in Table 1. As illustrated from the 9 individual experiments depicted in Table 1, the exposure of splenocytes to one of the 3 strains of probiotics reduced Treg activity by more than 50% and in some cases more than 80% (Experiments 6 and 9) at Treg:effector ratios of 1:8 and 1:4. For comparison, data with *L. plantarum* Lp-115 are included. As seen in the table, *L. plantarum* Lp-115 did not interfere with splenocyte-Treg activity.

Probiotic Cell Wall Is Responsible for Reduced Suppressor Activity of Tregs

In order to determine which part of the probiotic bacteria was responsible for the inactivating effect on Tregs we prepared the following 5 fractions of *L. acidophilus* NCFM, *B. bifidum* BI-98, and *L. plantarum* Lp-115: 1) plasmid DNA, 2) cell wall, 3) total DNA, 4) cytoplasm, and 5) protoplasts. $CD4^+CD25^-$ T cells were stimulated with fecal extract-pulsed splenocytes treated with the different probiotic fractions (equivalent to 100 μg/ml bacteria) during the process of enteroantigen-processing. As described above, the activation of $CD4^+CD25^-$ T cells was carried out in the presence of increasing numbers of Tregs (Figs. 3 and 4 for *L. acidophilus* NCFM and *B. bifidum* BI-98, respectively).

As observed above, both *L. acidophilus* NCFM and *B. bifidum* BI-98 caused a significant reduction in Treg activity (Figs. 3A and 4A). On the contrary, APC cultures not treated with probiotics were significantly inhibited by Tregs at Treg:effector ratios as low as 1:16 and upward.

Splenocytes exposed to *L. acidophilus* NCFM plasmid DNA, total DNA, cytoplasm, or protoplast fractions all showed a significant reduction in proliferation at Treg:effector ratios of 1:16 as seen for pulsed splenocytes; in contrast, the cell wall fraction only showed significant reduction in proliferation at Treg:effector ratios of 1:8 and upward (Fig. 3B). The same picture was seen for *B. bifidum* BI-98, where the plasmid DNA, total DNA, cytoplasm, and protoplast fractions all showed a significant reduction in proliferation at Treg:effector ratios of 1:8, thus not quite as effective as unfractionated *B. bifidum* BI-98. However, the cell wall fraction had the same Treg reducing profile as unfractionated *B. bifidum* BI-98, with a significant reduction in Treg activity at Treg:effector ratios of $<$1:4.

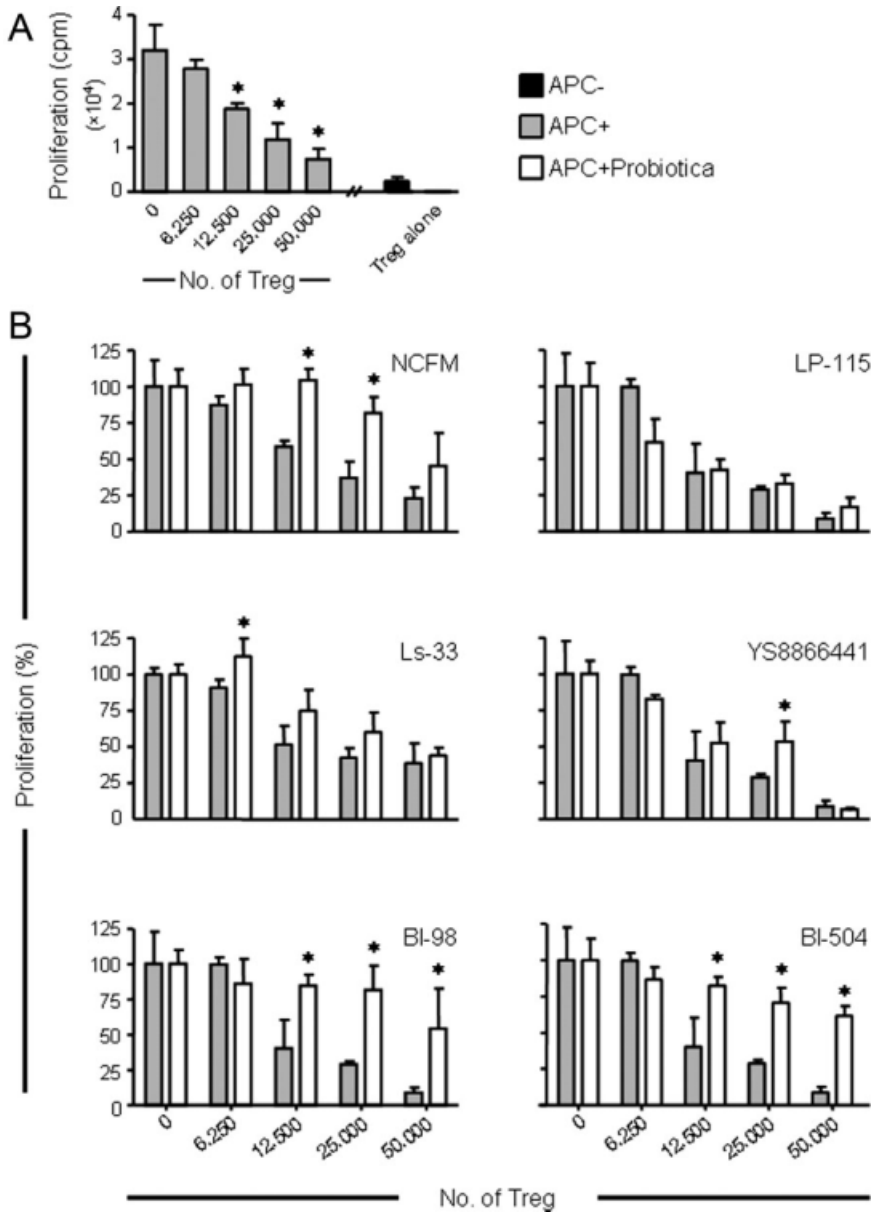

FIGURE 2. The inhibitory effect of Treg is reduced by splenocytes treated with probiotics. (A) Splenocytes pulsed with fecal extract (APC+) were cocultured for 5 days with $CD4^+CD25^-$ T cells in the presence of increasing numbers of Tregs. Proliferation was measured by ^{3}[H]-thymidine incorporation the last 18 hours of culture. Controls are proliferation of $CD4^+CD25^-$ T cells stimulated by unpulsed splenocytes (APC−) and proliferation of 50,000 Tregs cocultured with fecal extract-pulsed splenocytes (Treg alone). Error Bars: SD. *Significant Treg-mediated inhibition of proliferation, $P < 0.05$. (B) Proliferation of $CD4^+CD25^-$ T cells stimulated for 5 days by splenocytes, pulsed with fecal extract alone (gray bars) or in combination with probiotics (100 μg/mL) (white bars) in the presence of increasing numbers of Tregs. Proliferation is shown as percent of cultures without Tregs. Columns represent mean of quadruplicate cultures and for each probiotic strain 1 representative of at least 3 individual experiments is shown. Error bars: SD. *Significant reduction of Treg activity compared to the same treatment series without addition of probiotics, $P < 0.05$.

In order to rule out that probiotic cell wall in general has the ability to induce a reduction in Treg activity we also tested fractions of *L. plantarum* Lp-115, which do not have the ability to induce Treg inactivation (Fig. 2B, Table 1). As observed for intact *L. plantarum* Lp-115, none of these bacterial fractions induced a decrease in Treg activity (data not shown).

Reduction in Treg Activity Is Probiotic Dose-Dependent

$CD4^+CD25^-$ T cells were stimulated with fecal extract-pulsed splenocytes treated with increasing concentrations (0–100 μg/mL) of *L. acidophilus* NCFM, *B. bifidum* BI-98 or *B. bifidum* BI-504 during the process of enteroantigen-processing. The activation of $CD4^+CD25^-$ T cells was carried out in the absence of Tregs and at Treg:effector ratios of 1:8 (Fig. 5). As can be seen, the ability to induce reduction of Treg activity was dose-dependent and the inhibiting effect of Tregs was completely eliminated at the highest concentration of *L. acidophilus* NCFM, *B. bifidum* BI-98, and *B. bifidum* BI-504.

Treg Inactivation Is Not Mediated by IL-6 or IL-1β Release

The next line of experiments was carried out in order to identify the mechanisms behind the reduction in Treg

TABLE 1. Fecal Extract-Pulsed Splenocytes Exposed to *L. acidophilus* NCFM, *B. bififdum* BI-98, or *B. bififdum* BI-504 Reduce Treg-Mediated Suppression in Enteroantigen-Stimulated T cell Cultures

Exp. No.	Bacteria Strain	− Bacteria 1:8[a]	+ Bacteria 1:8[a]	− Bacteria 1:4[a]	+ Bacteria 1:4[a]
1	NCFM	39 ± 9	**77 ± 8**	30 ± 3	**61 ± 16 (2.0)**
2	NCFM	52 ± 13	**98 ± 11**	42 ± 7	**84 ± 10 (2.0)**
3	NCFM	59 ± 4	**104 ± 8**	37 ±11	**82 ± 11 (2.2)**
4	BI-98	40 ± 20	**85 ± 8**	29 ± 2	**82 ± 17 (2.8)**
5	BI-98	45 ± 6	**92 ± 25**	27 ± 6	**60 ± 15 (2.2)**
6	BI-98	45 ± 12	**96 ± 5**	6 ± 2	**44 ±14 (7.3)**
7	BI-504	40 ± 20	**83 ± 6**	29 ± 2	**71 ± 10 (2.5)**
8	BI-504	45 ± 6	**105 ± 22**	27 ± 6	**65 ± 21 (2.4)**
9	BI-504	45 ± 12	**90 ± 23**	6 ± 2	**69 ± 38 (11.5)**
10	Lp-115	40 ± 20	42 ±7	29 ± 2	33 ± 6
11	Lp-115	45 ± 6	37 ±10	27 ± 6	22 ± 5
12	Lp-115	45 ± 12	37 ±5	6 ± 2	10 ± 4

Splenocytes exposed to *L. Plantarum* Lp-115 do not reduce Treg activity. Treg-mediated suppression is shown as the percent of proliferation in corresponding cell cultures without Treg. Entries represent mean cpm values of 4 replicate cultures ± standard deviation values. All experiments in which the cultures included splenocytes exposed to the probiotic bacteria strains (bold) showed significantly decreased Treg activity (higher cpm values) compared with cultures without bacteria-exposed splenocytes (lower cpm values), $P \leq 0.05$, Wilcoxon test.
[a]Ratio between purified mesenteric lymph node $CD4^+CD25^+$ (Treg) and $CD4^+CD25^-$ (eff) T cells, respectively.

activity observed in cultures stimulated with splenocytes exposed to *B. bifidum* BI-98, *B. bifidum* BI-504, or *L. acidophilus* NCFM. Since it is well known that IL-6 efficiently reduces the function of Treg[37,38] we speculated that a probiotic-induced release of this cytokine from the splenocytes might have caused the reduced effect of Tregs in the T cell proliferation cultures. Of the 3 Treg-inactivating probiotic strains, *L. acidophilus* NCFM was chosen in the following experiments. First, the inhibitory effect of IL-6 on Treg activity was demonstrated in proliferation cultures, in which $CD4^+CD25^-$ T cells were stimulated with fecal extract-pulsed splenocytes not exposed to *L. acidophilus* NCFM (Fig. 6). As shown above, the presence of Tregs significantly reduced the level of proliferation by ≈40%. Addition of IL-6 resulted in complete abolition of Treg function and this effect of IL-6 was reversed by anti-IL-6 antibody (Ab). Thus, as described previously,[37] IL-6 reduces Treg function in the present culture system. As shown above, Treg function was reduced in cultures of *L. acidophilus* NCFM-exposed (100 μg/mL) splenocytes. However, the presence of anti-IL-6 Ab, at a concentration capable of blocking the Treg-reducing effect of IL-6, did not increase the activity of Tregs in these cultures (Fig. 6), indicating that IL-6 secretion by *L. acidophilus* NCFM-exposed splenocytes does not affect Treg function in this system. Similarly, we tested if the addition of anti-IL-1β antibody in the test system would affect the function of Treg in fecal extract-pulsed splenocytes exposed to *L. acidophilus* NCFM. As for anti-IL-6 Ab, anti-IL-1β Ab did not increase Treg activity in these cultures (data not shown).

Altered Cytokine Profile of Splenocyte Culture Supernatants

Supernatants from fecal extract-pulsed splenocyte cultures not exposed or exposed for 24 hours to *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 were tested for the presence of IL-6, IL-1β, and IL-10, by ELISA analysis (Fig. 7). The concentration of IL-6 was comparable in all cultures, thus supporting our findings that IL-6 is not responsible for the observed reduction in Treg activity. In contrast, the concentrations of both IL-1β and IL-10 were significantly increased in the cultures exposed to *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 compared to the fecal extract-pulsed culture. Also, the *L. acidophilus* NCFM culture contained significantly higher amounts of IL10 than all the other cultures. The 2 *B. bifidum*-exposed cultures contained significantly more IL-1β than either the *L. acidophilus* NCFM exposed and non-exposed splenocyte culture.

IL-10 Concentration in Proliferation Culture Treated with *L. acidophilus* NCFM, *B. Bifidum* BI-98, or *B. Bifidum* BI-504

We also measured the levels of IL-6, IL-1β, and IL-10 in T cell proliferation cultures, in which $CD4^+CD25^-$ T cells were stimulated with fecal extract-pulsed splenocytes unexposed or exposed to *L. acidophilus*

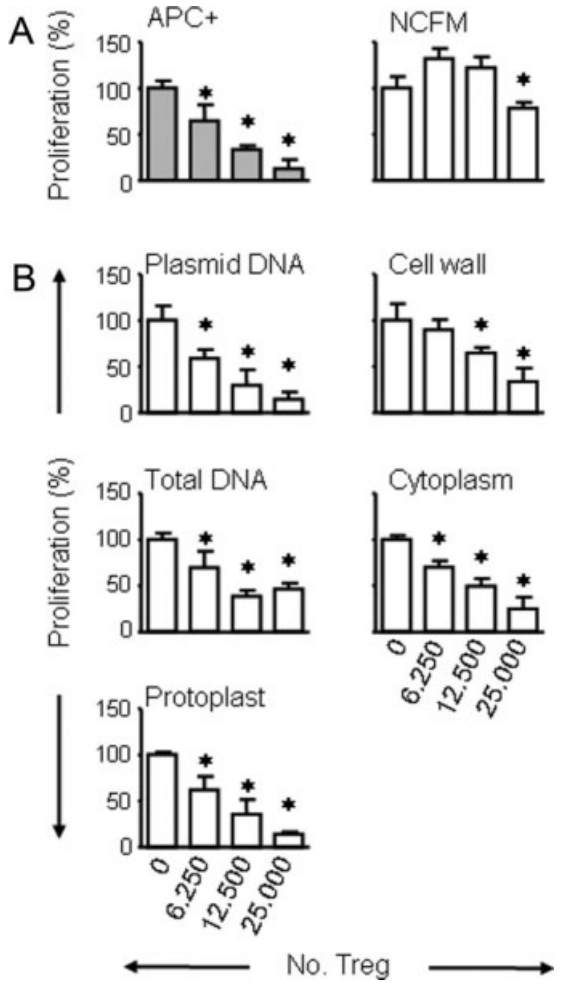

FIGURE 3. *L. acidophilus* NCFM cell-wall reduces Treg activity. (A) Splenocytes were pulsed with fecal extract alone (APC+) or in combination with *L. acidophilus* NCFM (100 μg/mL) and subsequently cocultured with 10^5 $CD4^+CD25^-$ T cells and increasing numbers of Tregs. Proliferation of cultures with Tregs is shown as percent of cultures without Tregs and measured after 5 days. (B) Splenocytes were pulsed with fecal extract in combination with different cellular fractions of *L. acidophilus* NCFM (equivalent to 100 μg/mL bacteria) and cocultured with $CD4^+CD25^-$ T cells and Tregs as in A. Each bar represents mean of quadruplicate cultures. One representative experiment of 3 is shown. Error bars: SD. *Significant Treg-induced reduction in proliferation compared to 0 Treg, $P < 0.05$.

NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 and with increasing numbers of Tregs (Fig. 8). As observed in the splenocyte culture supernatants (see above), there were significantly increased levels of IL-10 in the cultures of T cells stimulated with splenocytes exposed to the 3 probiotic strains. This being significantly higher in the 2 cultures stimulated with *B. bifidum*-exposed splenocytes compared to the *L. acidophilus* NCFM, which is contrary to IL-10 levels in the splenocyte cultures. IL-10 secretion seems to be independent of Tregs, as the level was also increased in the culture with no Tregs.

The concentrations of IL-6 and IL-1β in the T cell cultures were in general very low (less than 200 pg/mL in all samples) and there were no notable differences between the cultures of splenocytes not exposed or exposed to the 3 probiotic strains (data not shown).

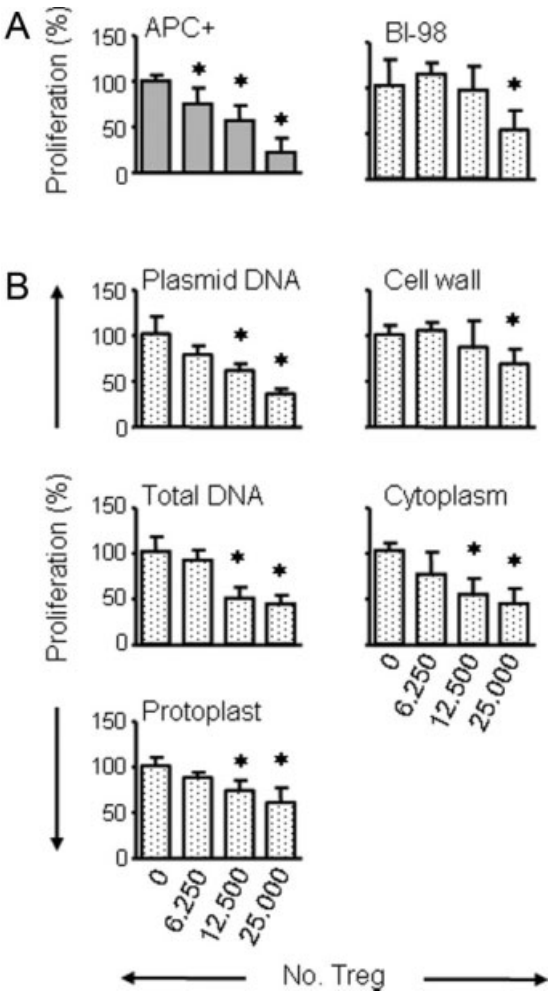

FIGURE 4. *B. bifidum* BI-98 cell-wall reduces Treg activity. (A) Splenocytes were pulsed with fecal extract alone (APC+) or in combination with *B. bifidum* BI-98 (100 μg/mL) and subsequently cocultured with 10^5 $CD4^+CD25^-$ T cells and increasing numbers of Tregs. Proliferation of cultures with Tregs is shown as percent of cultures without Tregs and measured after 5 days. (B) Splenocytes were pulsed with fecal extract in combination with different cellular fractions of *B. bifidum* BI-98 (equivalent to 100 μg/mL bacteria) and cocultured with $CD4^+CD25^-$ T cells and Tregs as in A. Each bar represents mean of quadruplicate cultures. One representative experiment of 3 is shown. Error bars: SD. *Significant Treg-induced reduction in proliferation compared to 0 Treg, $P < 0.05$.

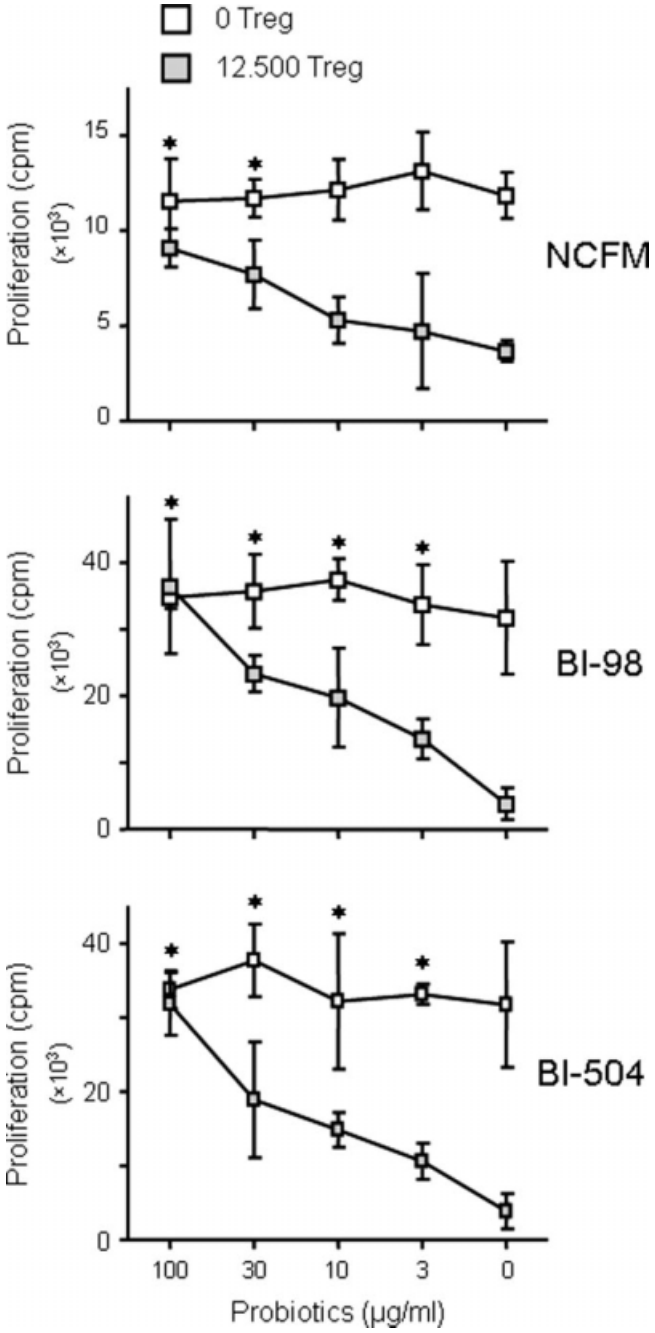

FIGURE 5. *L. acidophilus* NCFM, *B. bifidum* BI-98, and *B. bifidum* BI-504 reduces the activity of Treg in a dose-dependent manner. 10^5 $CD4^+CD25^-$ T cells were stimulated by splenocytes, pulsed with fecal extract (APC+) and decreasing concentrations of *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 (100-0 μg/mL), in the presence of ±12,500 Treg. Proliferation was measured the last 18 hours of 5 days coculture. Each data point represents the mean of quadruplicate cultures. One representative of 3 is shown. Error bars: SD. *Significant *probiotic*-dose-dependent decrease in Treg activity, $P < 0.05$.

DISCUSSION

The major observation of the present work is that splenocytes pulsed with a fecal extract and exposed to 3 of 6 studied probiotic bacteria (*L. acidophilus* NCFM, *B. bifidum* BI-98, and *B. bifidum* BI-504) reduce the suppressor activity of Tregs during stimulation of enteroantigen-specific T cells. The mechanisms responsible for this inhibitory effect on Tregs are at present unknown. First, we speculated that Treg-inhibiting lactic acid bacterial strains would increase the stimulatory capacity of enteroantigen-loaded splenocytes per se. However, the data (Fig. 1B) did not support this hypothesis since splenocyte-induced T cell stimulation was independent of exposure to the 6 probiotic strains under study. Second, we speculated that the proinflammatory cytokines IL-6 and/or IL-1β released from splenocytes exposed to the lactic acid bacteria render the responder T cells refractory to the suppressive effect of

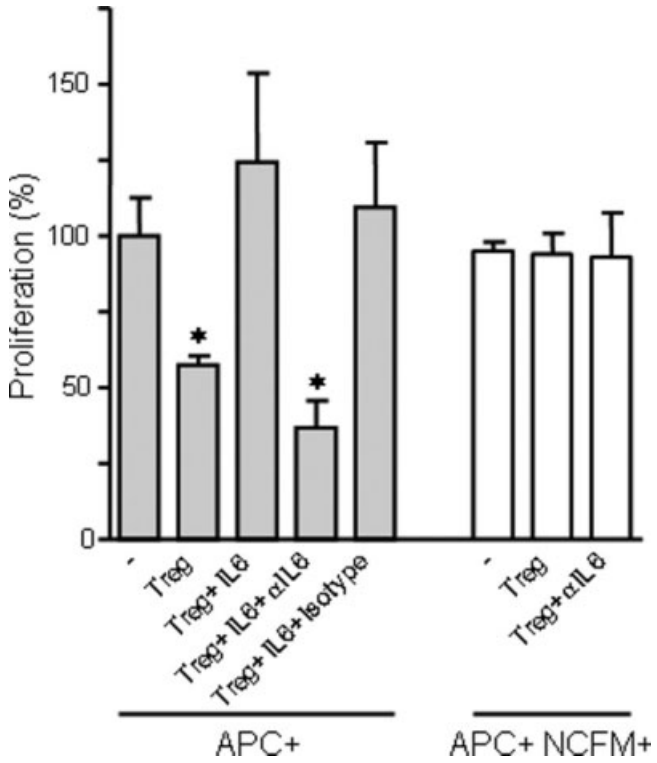

FIGURE 6. The *L. acidophilus* NCFM-induced inhibitory effect on Tregs is not dependent on IL-6. Splenocytes pulsed with fecal extract alone (gray bars) or in combination with *L. acidophilus* NCFM (100 μg/mL) (white bars) were cocultured with 10^5 $CD4^+CD25^-$ T cells. The stimulation-cultures received either nothing (--) or 12,500 Tregs, IL-6 (5 ng/mL), anti-IL-6 mAb, an mAb isotype control or combinations of these. After 5 days the proliferation was measured and is shown as percent of (--) cultures. Each column represents the mean of quadruplicate cultures and 1 of 3 representative experiments is shown. Error bars: SD. *Significant Treg activity, $P < 0.05$.

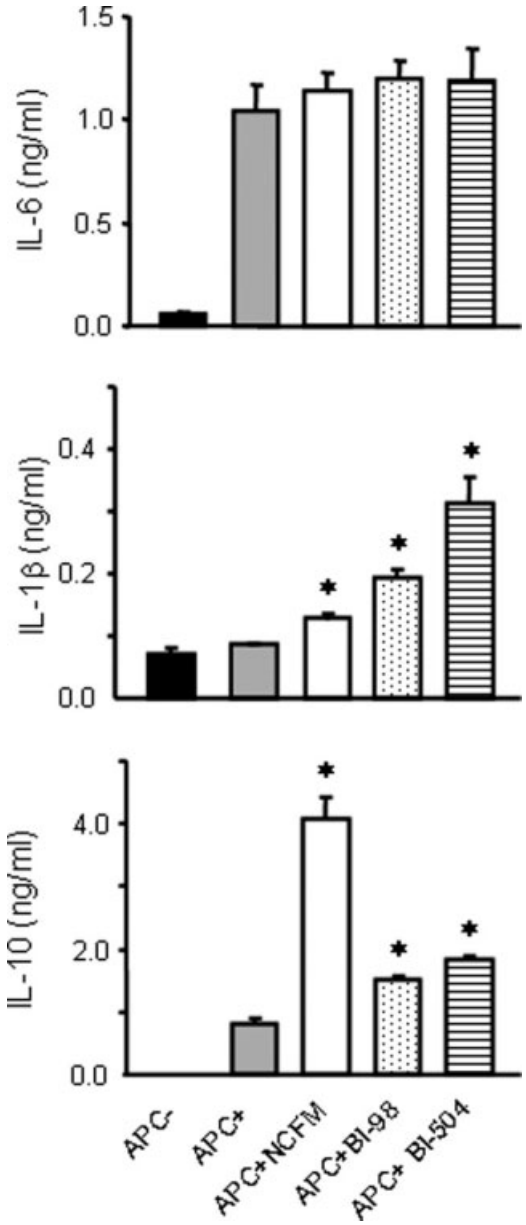

FIGURE 7. Secretion of IL-1β and IL-10 by splenic APCs is upregulated by *L. acidophilus* NCFM, *B. bifidum* BI-98, and *B. bifidum* BI-504. Splenocytes were left unpulsed (APC−) or pulsed with fecal extract alone (APC+) or in combination with *L. acidophilus* NCFM-, *B. bifidum* BI-98, or *B. bifidum* BI-504 (100 μg/mL) as indicated for 24 hours. Supernatants were collected and concentrations of IL-6, IL-1β, and IL-10 were measured by ELISA. Each column represents mean of triplicate measurements and 1 of 3 independent experiments are shown. Error bars: SD.

Treg,[37–39] thereby being responsible for Treg inhibition. But experiments including both biological levels of IL-6 added to the culture system and antibody-mediated IL-6 and IL-1β blocking clearly demonstrated that neither of these 2 cytokines are involved in the inhibition of Treg activity in our experimental model. Furthermore, we showed that the presence of *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 during antigen-processing changes the cytokine profile of the culture to be dominated by IL-10 rather than IL-6 and IL-1β. In the T cell culture stimulated with these APCs only very low levels of IL-6 and IL-1β were present, confirming that these 2 cytokines are not responsible for the inactivating effect seen on Tregs. Thus, these data suggest indirectly that Treg inhibition is mediated by direct cell-to-cell contact between the splenocytes and the Tregs. The T cell proliferation culture stimulated with *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504-exposed splenocytes also show increased levels of IL-10 independently of the presence of Tregs.

The molecular interactions between bacteria and APCs are mediated by pathogen-associated molecular patterns (PAMPs) including bacterial lipoteichoic acid, peptidoglycans (PGNs), and lipoproteins that bind to pattern recognition receptors (PRRs) on APCs such as Toll-like receptors (TLRs) and NOD2. Such binding facilitates recognition of lipids, carbohydrates, proteins, and nucleic acids expressed by various microorganisms. It has previously been documented that signaling through TLRs and NOD receptors leads to T_H1 and T_H2 polarizing activity of the APCs.[40–44] The interactions between APCs and bacteria and the internalization of lactic acid bacteria may induce either T_H1 or T_H2 polarization, depending on the actual bacterial strain or combinations of such strains.[45] Thus, some strains of lactic acid bacteria bind via bacterial expressed lipoteichoic acid and peptidoglycan to TLR2[46]

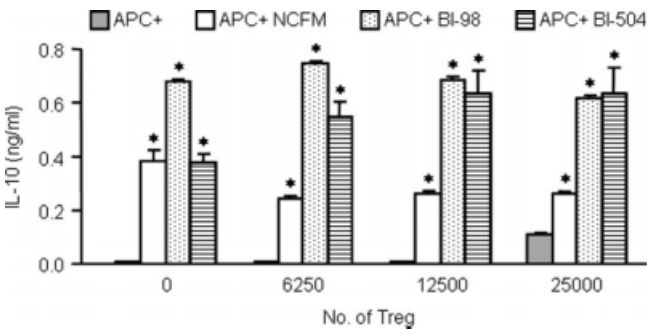

FIGURE 8. *L. acidophilus* NCFM-, *B. bifidum* BI-98-, and *B. bifidum* BI-504-treated splenocytes cause upregulation of IL-10 in T cell proliferation cultures independent of Treg concentration. Splenocytes pulsed with fecal extract alone (APC+) or in combination with *L. acidophilus* NCFM, *B. bifidum* BI-98, or *B. bifidum* BI-504 (100 μg/mL) (as indicated) were cocultured with 10^5 $CD4^+CD25^-$ T cells in presence of increasing numbers of Tregs. After 3 days stimulation supernatants were collected and concentrations of IL-10 were measured by ELISA. Each column represents the mean of triplicate measurements and 1 of 3 independent experiments are shown. Error bars: SD.

and NOD2,[47] respectively, in this way activating the NF-κB inflammatory pathway with TNFα and IL-12 secretion. In contrast, bifidobacteria and some strains of lactobacilli induce Treg-polarizing dendritic cells when bacterial expressed lipoprotein binds to TLR2 in complex with TLR1 or TLR6, thereby initiating signals that prevent peptidoglycan-induced activation of NF-κB and secretion of the inflammatory cytokines TNFα and IL-12.[48] In all cases, internalization of bacteria by dendritic cells probably depends on their binding to the C-lectin-like receptor DC-SIGN.[49] Since the bacterial cell wall fraction appears to have almost the same activity as intact bacteria on APC-mediated suppression of Treg activity, it is likely that PGNs and lipoproteins located in the outer layers of the cell wall are the active bacterial components in *L. acidophilus* NCFM, *B. bifidum* BI-98, and *B. bifidum* BI-504. Thus, at the APC level both PGN activating the NOD2 pathway and LP activating the TLR2 in complex with CD14, CD36, and TLR1, or TLR6 might be involved.[50–53]

Various compositions of lactic acid bacteria are currently in use to alleviate symptoms in patients suffering from IBD and allergic diseases.[54,55] Generally, the effect of the probiotic bacteria in these patients is assumed to be antiinflammatory in nature, an effect that might include recruitment of Tregs to the site of inflammation. However, in the light of the present observations we find it important to stress that some lactic acid bacterial strains may reduce instead of increase Treg activity, thereby interfering negatively with the antiinflammatory condition. Therefore, we find it of importance that in vitro assays are used to test the Treg-reducing activity of the individual lactic acid bacterial strains prior to their clinical use in patients.

In a pilot study by Paineau et al[56] it was demonstrated that probiotics together with orally administrated cholera vaccine results in a more rapid onset of the specific immune response. Thus, lactic acid bacteria might be promising adjuvants in vaccines due to their capability to reduce functional activity of Tregs, thereby speeding up vaccine-induced immune responses. Particularly in cancer vaccines, a Treg-reducing adjuvant would be of importance to potentiate the immune response against the tumor, since these vaccines appear to be constrained by the increased Treg activity in cancer patients.[57]

In conclusion, the functional activity of Tregs in antigen-specific T cell proliferation assays is reduced if the antigen-presenting cells are exposed to certain strains of probiotics, including both lactobacilli and bifidobacteria. This observation should be taken into consideration when designing adjuvant therapies with probiotics.

ACKNOWLEDGMENTS

The authors thank Dr. Arthur Ouwehand for providing the probiotics strain *Lactobacillus acidophilus* NCFM, *Lactobacillus salivarius* Ls-33, and *Lactobacillus plantarum* Lp-115. *B. bifidum* BI-98 and *B. bifidum* BI-504 were isolated and characterized at Bioneer by Kim Holmström and Trine Möller. We also thank Bente Thorup for valuable technical assistance.

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