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Lactobacilli Differentially Modulate Expression of Cytokines and Maturation Surface Markers in Murine Dendritic Cells[1]

Hanne R. Christensen,*†‡ Hanne Frøkiær,‡ and James J. Pestka[2]*†

Dendritic cells (DC) play a pivotal immunoregulatory role in the Th1, Th2, and Th3 cell balance and are present throughout the gastrointestinal tract. Thus, DC may be targets for modulation by gut microbes, including ingested probiotics. In the present study, we tested the hypothesis that species of *Lactobacillus*, important members of the gut flora, differentially activate DC. Bone marrow-derived murine DC were exposed to various lethally irradiated *Lactobacillus* spp. and resultant culture supernatants were analyzed for IL-6, IL-10, IL-12, and TNF-α. Substantial differences were found among strains in the capacity to induce IL-12 and TNF-α production in the DC. Similar but less pronounced differences were observed among lactobacilli in the induction of IL-6 and IL-10. Although all strains up-regulated surface MHC class II and B7-2 (CD86), which is indicative of DC maturation, those lactobacilli with greatest capacity to induce IL-12 were most effective. Remarkably, *Lactobacillus reuteri* DSM12246, a poor IL-12 inducer, inhibited IL-12, IL-6, and TNF-α induction by the otherwise strong cytokine inducer *L. casei* CHCC3139, while IL-10 production remained unaltered. In analogous fashion, *L. reuteri* reduced *L. casei*-induced up-regulation of B7-2. These results suggest that different species of *Lactobacillus* exert very different DC activation patterns and, furthermore, at least one species may be capable of inhibiting activities of other species in the genus. Thus, the potential exists for Th1/Th2/Th3-driving capacities of the gut DC to be modulated according to composition of gut microflora, including ingested probiotics. ***The Journal of Immunology*, 2002, 168: 171–178.**

Lactobacilli are a major component of the commensal microbial flora of both the small and large intestinal tract of humans and animals and are frequently used as probiotics (1). Probiotics are defined as "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" (2). It is widely accepted that the intestinal microflora play an important role for the health of the host and possess immunomodulating capacity. Elucidation of the mechanisms by which intestinal microorganisms, including potential probiotics, modulate the immune system may facilitate implementation of therapeutic supplements of probiotics that are individually tailored immunoregulatory properties.

One potentially important immunoregulatory function of the intestinal microflora is their involvement in generation of immunocompetent cells during development and maintenance of homeostasis of the gut-associated immune system (3, 4). For example, although the neonatal immune response is initially characterized by a Th2 cell cytokine profile, bacterial stimuli emerging from the postnatal gut colonization apparently play a major role in driving this Th2-skewed immune response toward a more finely balanced Th1 and Th2 immune response. Studies of this counterbalancing effect of intestinal bacteria on the Th2 polarized stage in germ-free newborn rodents have revealed an important role of the bacteria in rendering the animals fully susceptible for oral tolerance induction (5). Although, to date, no bacterial species has been found to be uniquely involved in these processes, lactobacilli are potential candidates because many species of *Lactobacillus*, including the species used in the present study, have been demonstrated to be immunomodulatory in vitro and in vivo (6, 7). Very recent clinical studies in both human infants and adults have shown an association between administration of a strain of *Lactobacillus* and alleviation of intestinal inflammation caused by food allergy (8, 9). Moreover, studies of composition of the intestinal flora have indicated a tendency of allergic children to be less often colonized with lactobacilli compared with nonallergic children (3, 10).

In clarifying the mechanisms behind the immunoregulatory effect of the gut flora, including probiotic bacteria, relatively little attention has been focused on their effect on dendritic cells (DC).[3] DC are distributed in most tissues and, in particular, at sites that interface with external environment, such as the mucosa of the gastrointestinal tract, where they reside in the Peyer's patch, lamina propria, and draining mesenteric lymph nodes (11). DC are the gatekeepers of an immune response and are the principal stimulators of naive Th cells—a property that distinguishes them from all other APCs (12). It has been widely suggested that the delicate balance between Th1 and Th2 immunity, as well as tolerance (Th3), is pivotally controlled by the stimulating DC (13, 14). However, in executing any of the three distinct functions, Th1, Th2, or Th3 priming of naive T cells, it remains unclear whether specific activation states of the DC are responsible for each of the different responses or whether there are distinct lineages of DC predisposed to direct each of the responses.

Departments of *Microbiology and Molecular Genetics and †Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824; and ‡BioCentrum-DTU, Section for Biochemistry and Nutrition, Technical University of Denmark, Lyngby, Denmark

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[2] Address correspondence and reprint requests to Dr. James J. Pestka, Departments of Microbiology and Molecular Genetics and Food Science and Human Nutrition, Michigan State University, 234 G. M. Trout Building, East Lansing, MI 48824. E-mail address: pestka@msu.edu

[3] Abbreviations used in this paper: DC, dendritic cell; TLR, Toll-like receptor; MRS, de Man, Rogosa, and Sharpe broth.

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Recent studies have demonstrated the occurrence of tissue-specific differences among DC in activating Th cells; DC isolated from mucosal tissues preferentially induce a Th2 phenotype, whereas splenic DC induce a Th1 phenotype (15, 16). Other recent studies have revealed that intestinal DC can either be tolerogenic or immunogenic, depending on their type and state of activation (17, 18). The ability of activated DC to prime T cell activity is, in addition to expression of MHC Ag complexes, attributable to DC expression of cytokines and costimulatory molecules that are upregulated during maturation. The discriminative factors in this respect are production of the strong Th1-skewing cytokine IL-12 as well as the expression of the costimulatory molecules B7-1 (CD80) and especially B7-2 (CD86) (17–24). DC inhabiting the gut mucosa are constantly in close proximity to a microenvironment consisting of microorganisms in abundance. Thus, it appears reasonable that the intestinal flora including probiotics may exert immunoregulatory effects through modulation of the Th1/Th2/Th3-promoting capacity of the DC in the gut.

In view of the critical importance of DC polarization in the regulation of the immune response, the objective of the present study was to examine the effect of selected strains of *Lactobacillus* on activation patterns of murine DC. The results suggest that different species of *Lactobacillus* exhibited very different polarization patterns when activating DC in vitro. Interestingly, one species possessed the capability to selectively suppress the otherwise strong type 1 DC-polarizing effect exerted by some species.

Materials and Methods

Lactobacillus *cultures*

Six lactobacilli strains were used in this study, including *Lactobacillus reuteri* DSM12246, *Lactobacillus plantarum* Lb1, *Lactobacillus fermentum* Lb20 (kindly supplied by Department of Dairy and Food Science at the Royal Veterinary and Agricultural University, Frederiksberg, Denmark), *Lactobacillus casei* subsp. *alactus* CHCC3137 (Chr. Hansen, Hørsholm, Denmark), *Lactobacillus plantarum* 299v (Probi, Lund, Sweden), and *Lactobacillus johnsonii* La1 (Nestlé Research Center, Lausanne, Switzerland). From frozen stocks (−80°C), bacteria were inoculated in de Man, Rogosa, and Sharpe broth (MRS; Difco, Detroit, MI) and grown at 37°C for 15 h. From this culture, 10 ml were transferred to 500 ml of fresh MRS and incubated at 37°C until mid-log phase (6–10 h). Bacteria were collected by centrifugation at 1000 × *g* for 15 min at 4°C, washed three times in 0.01 M PBS (pH 7.2), resuspended in PBS at one-tenth their original volume, and then frozen immediately at −80°C. The frozen bacterial cells were killed by gamma irradiation (Phoenix Memorial Laboratory Cobalt-60 Irradiation Facility, University of Michigan, Ann Arbor, MI) using a dose of 1 Mrad. Absence of viable cells was verified by plate counts on MRS medium. Dry weight of cultures was determined by freeze-drying aliquots and correcting for buffer salt content.

Bone marrow cell culture

Bone marrow cells were isolated and cultured as described by Lutz et al. (25), with minor modifications. Briefly, femora and tibiae from two female C57BL/6 mice, 8–12 wk (Charles River Breeding Laboratories, Portage, MI), were removed and stripped of muscles and tendons. After soaking the bones in 70% ethanol for 2 min and rinsing in PBS, both ends were cut with scissors and the marrow was flushed with PBS using a 27-gauge needle. Cell clusters were dissociated by repeated pipetting. The resulting cell suspension was centrifuged for 10 min at 300 × *g* and washed once in PBS.

Cells were resuspended in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 10% (v/v) heat-inactivated FBS (Atlanta Biologicals, Norcross, GA), and 15 ng/ml murine GM-CSF. GM-CSF was added as 5–10% (v/v) culture supernatant harvested from a GM-CSF-producing cell line (GM-CSF transfected Ag8.653 myeloma cell line) (26). The GM-CSF-producing cell line was kindly provided by Dr. R. Tisch (University of North Carolina, Chapel Hill, NC) and GM-CSF-quantified using a specific ELISA kit (BD PharMingen, San Diego, CA).

To enrich for DC, 10 ml of cell suspension containing 3×10^6 leukocytes was seeded per 100-mm bacteriological petri dish (day 0) and incubated for 8 days at 37°C in 5% CO_2. An additional 10 ml of freshly prepared medium was added to each plate on day 3. On day 6, 9 ml from each plate was centrifuged for 5 min at 300 × *g*, resultant cell pellet was resuspended in 10 ml of fresh medium, and the suspension was returned to the dish. On day 8, cells were used to evaluate effects of lactobacilli on cytokine release and expression of surface markers as described below.

Induction of cytokine release

Nonadherent cells were gently pipetted from petri dishes containing 8-day old DC-enriched cultures. The collected cells were centrifuged for 5 min at 300 × *g* and resuspended in medium supplemented with only 10 ng/ml GM-CSF. Cells were seeded in 48-well tissue culture plates at 1.4×10^6/500 μl/well. Medium containing irradiated lactobacilli (1–100 μg/ml) were then added at 100 μl/well. LPS (*Escherichia coli* O26:B6; Sigma-Aldrich) at 1 μg/ml was added to some cultures as positive control. Medium alone or medium containing 2-μm latex beads (Polysciences, Warrington, PA) were used as unstimulated and negative controls, respectively. After a stimulation period of 15 h at 37°C in 5% CO_2, culture supernatant was collected and stored at −80°C until cytokine analysis.

Cytokine quantification in culture supernatants

IL-12(p70) and TNF-α were analyzed using commercially available ELISA kits (BD PharMingen) according to manufacturer's instructions. IL-10 and IL-6 were similarly analyzed using matched Ab pairs purchased from BD PharMingen.

Induction of surface markers

A total of 10 ml of the top medium was carefully removed and discarded from 8-day old DC-enriched cultures in petri dishes (described above), each containing 20 ml. Then, 10 ml of medium without GM-CSF in the absence (unstimulated) or the presence of irradiated lactobacilli (1–100 μg/ml) or LPS (1 μg/ml) was added to each dish. Medium containing 2-μm latex beads was used as negative control. Because medium containing no GM-CSF was used, the final concentration of GM-CSF in the culture was reduced to 7.5 ng/ml during the stimulation period. Cultures were incubated for 12 h at 37°C in 5% CO_2. At the end of the incubation period, 3 ml of PBS containing 1% (v/v) FBS and 0.15% (w/v) sodium azide (PBS-Az) was added to each petri dish to prevent internalization of surface markers during subsequent handling of cells. Nonadherent cells were collected by gentle pipetting, centrifuged for 5 min at 300 × *g*, and subjected to surface marker staining for flow cytometry analysis as described below. Trypan blue indicated that the lactobacilli-treated stimulated cultures contained ≥95% viable cells.

Immunocytostaining and flow cytometry

Centrifuged cells were resuspended in cold PBS-Az and transferred to a round-bottom 96-well plate at 4×10^5 cells/well. During all work, cells were kept at or below 4°C and at low light exposure. Cells were centrifuged and incubated 10 min in 50 μl/well PBS-Az containing anti-mouse FcγRII/III (3 μg/ml; BD PharMingen) to block nonspecific binding of Ab reagents. After incubation, an additional 50 μl/well of PBS-Az containing fluorochrome-conjugated Abs at 10 μg/ml was added and incubated for 45 min. Thereafter, cells were washed twice in 200 μl/well PBS-Az and finally resuspended in 300 μl/well PBS-Az for flow cytometric analysis in a BD FACSVantage SE flow cytometer (BD Biosciences, San Jose, CA). The analysis was based on counting 20,000 cells. The following Abs used for staining were purchased from BD PharMingen: PE-conjugated anti-mouse CD11c, clone HL3 (hamster IgG); FITC-conjugated anti-mouse MHC II (I-A/I-E), clone 2G9 (rat IgG2b); PE-conjugated anti-mouse B7-2, clone GL1 (rat IgG2a); PE-conjugated anti-mouse B220, clone RA3-6B2; and isotype controls for hamster IgG, clone G235-2356; rat IgG2b, clone A95-1; rat IgG2a, clone R35-95.

Statistical analysis

One-way ANOVA with experiments assigned as blocks was applied to reveal significant differences between bacterial species relative to capacity to induce cytokine production in three independent experiments. Statistical calculations were performed with GraphPad software, version 3.02 (GraphPad, San Diego, CA).

Results

Lactobacillus *spp. differentially induce IL-6, IL-10, IL-12, and TNF-α*

To assess the effect of the various species of *Lactobacillus* on cytokine production in DC, three different concentrations of whole washed, irradiated bacteria (1, 10, and 100 μg/ml) were added to

DC cultures and IL-12, IL-6, IL-10, and TNF-α were measured in culture supernatants (Fig. 1). LPS at 1 μg/ml was included as a positive control in all experiments. Supernatant was collected after 15 h, which was found optimal in preliminary kinetic analyses (data not shown). Lactobacilli induced production of all cytokines in a dose-dependent fashion. However, the bacterial concentration giving rise to the highest level of IL-12, IL-6, and TNF-α ($\sim$10 μg/ml) was much lower than the bacterial concentration inducing the highest level of IL-10 ($\sim$100 μg/ml). Increasing the bacterial concentration to 300 μg/ml did not further increase the level of IL-10 (data not shown). Thus, while a concentration of 10 μg/ml *L. casei* induced a high level of IL-12, IL-6, and TNF-α, but almost no IL-10, 100 μg/ml induced the same level of IL-12 but a much greater level of IL-10. These data indicate a dose-dependent outcome of not only the individual levels of produced cytokine but also the cytokine pattern, as the ratio between the cytokine levels varied with the bacterial concentration.

The most significant differences among the tested lactobacilli species were observed for IL-12 and TNF-α (Fig. 1, *A* and *D*, respectively), with the following ranking of the species: *L. casei* $\gg$ *L. plantarum* Lb1 > *L. fermentum* Lb20 $\sim$ *L. johnsonii* La1 $\sim$ *L. plantarum* 299v $\gg$ *L. reuteri*; cytokine responses ranged from no induction observed for *L. reuteri* to responses induced by *L. casei* far exceeding that achieved by LPS stimulation. Similar but less pronounced patterns were observed for IL-6 and IL-10 (Fig. 1, *B–C*). Interestingly, *L. reuteri* exhibited a very weak capacity to induce IL-12 production yet showed a moderate potential to induce these other cytokines, thus further indicating a species- and dose-dependent outcome of the induced cytokine profile.

To test the effect per se of inert particles by DC on induction of cytokine production, DC was incubated with 2-μM latex beads (10^8/ml), which induce phagocytosis in DC (27). Microscopic inspection of the DC after incubation with particles clearly revealed that phagocytosis of the particles had taken place. However, phagocytosis of particles by DC did not induce cytokine levels that exceeded unstimulated DC (data not shown). Thus, simple phagocytosis of the lethally irradiated bacteria particles alone was likely not sufficient to induce cytokine production.

L. reuteri *inhibits* L. casei*-induced IL-12, IL-6, and TNF-α production*

Based on the finding that *L. reuteri* is a poor inducer of IL-12, TNF-α, and IL-6 when compared with *L. casei*, the effect of *L. reuteri* on the *L. casei*-induced cytokine release was assessed. Specifically, DC cultures were exposed to mixtures of varying concentrations of *L. reuteri* and *L. casei*. *L. reuteri* markedly inhibited *L. casei*-induced IL-12, IL-6, and TNF-α production (Fig. 2, *A*, *C*, and *D*, respectively). The inhibition was dose-dependent, with maximum inhibition corresponding to a lower limit of cytokine production at the level induced by *L. reuteri* alone, indicating a complete abrogation of the *L. casei* effect. *L. casei* induction of IL-10 was unaffected by *L. reuteri*; instead, combinations of *L. reuteri* with *L. casei* or LPS exhibited an additive effect on IL-10 induction (Fig. 2*B*). Interestingly, LPS induction of IL-12, IL-6,

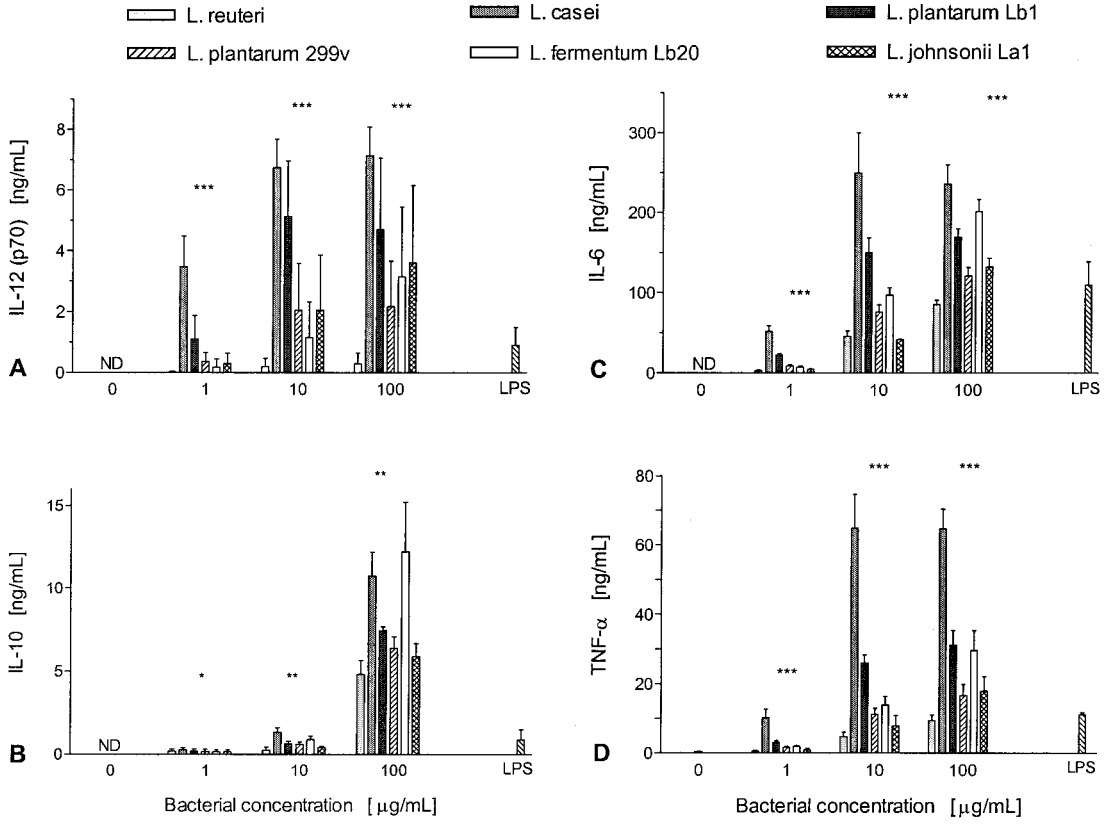

FIGURE 1. Lethally irradiated lactobacilli induce cytokine production in DC cultures. Cytokines IL-12(p70) (*A*), IL-10 (*B*), IL-6 (*C*), and TNF-α (*D*) were analyzed by ELISA in supernatants collected from 8-day cultures of murine bone marrow-derived DC cultured for an additional 15 h in 48-well plates at 1.4×10^6/600 μl/well with different concentrations of bacteria or LPS (1 μg/ml). Bacterial concentrations ranged between 0 (unstimulated controls) and 100 μg/ml of dry material. Data are mean $\pm$ SEM values derived from three independent experiments, each based on DC derived from two mice and tested in duplicate wells. ND, Not detectable. *, Significant differences between bacterial species within each concentration level tested by one-way ANOVA (*, $p \leq 0.05$; **, $p \leq 0.01$; *** $p \leq 0.001$).

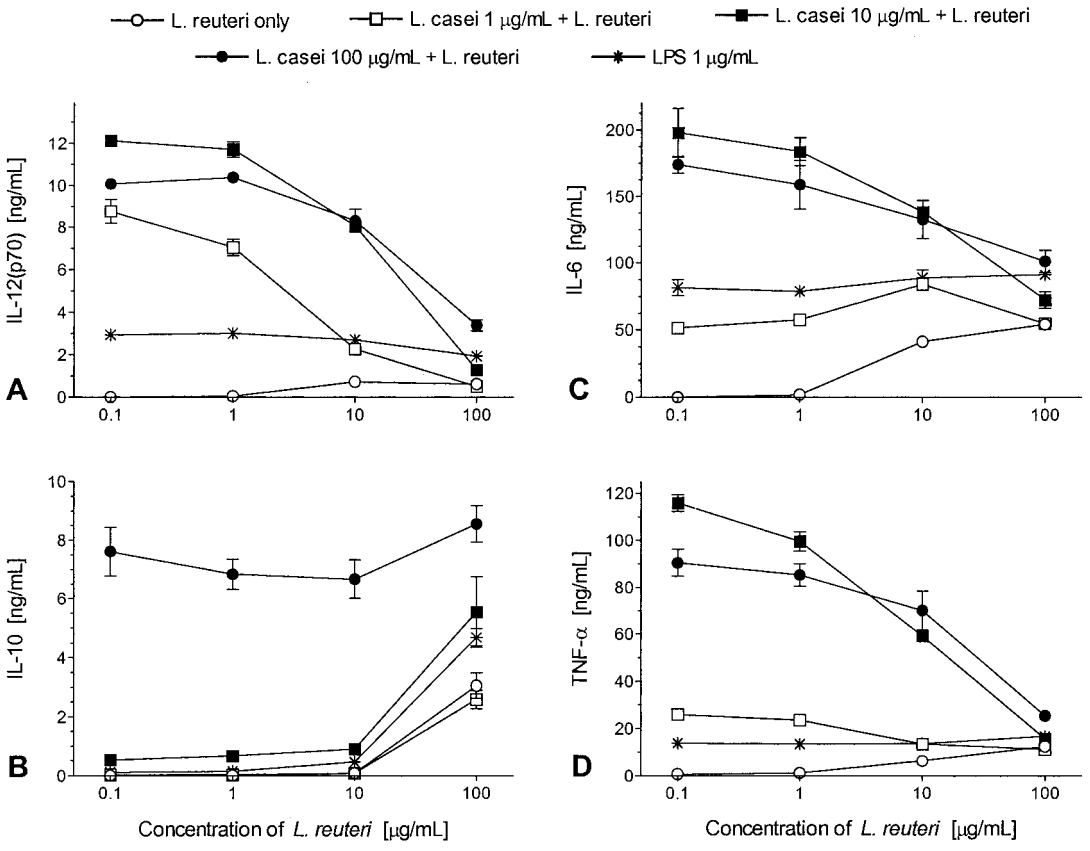

FIGURE 2. *L. reuteri* inhibits *L. casei*-induced cytokine production in DC. Cytokines IL-12(p70) (*A*), IL-10 (*B*), IL-6 (*C*), and TNF-α (*D*) were analyzed by ELISA in supernatants collected from DC cultures exposed to combinations at varying concentrations of *L. reuteri* and *L. casei* or LPS. The results shown are representative of two experiments performed. Analogous experiments with *L. johnsonii* showed similar results (data not shown). Error bars represent SD.

TNF-α, or IL-10 was refractory to coincubation of *L. reuteri* at any concentration (Fig. 2, *A–D*). Analogous trials involving combinations of *L. johnsonii* La1, a moderate IL-12 inducer (Fig. 1), and *L. reuteri* exhibited identical patterns of inhibition as described above for *L. casei* and *L. reuteri* (data not shown).

Lactobacillus *spp. differentially up-regulate maturation surface marker expression*

Upon culturing murine bone marrow cells with GM-CSF, cells other than DC can develop, such as myeloid progenitors, granulocytes, macrophages, and lymphocytes. Therefore, DC purity was verified by flow cytometry using the DC marker CD11c. In day 9 cultures, the amount of $CD11c^+$ cells was in the range of 85–90%. The amount of contaminating B cells, detected as $B220^+$ cells expressing high or low levels of MHC class II, was ~5–6%. By microscopic inspection, the vast majority of the cells appeared as irregularly shaped cells with clearly visible protrusions, which are characteristic morphological features for DC. The obtained level of DC purity in the cultures is consistent with results from previous studies (25).

Based on the level of MHC class II expression on the cell surface, immature and mature DC subsets can be recognized as MHC class II^{low} and MHC class II^{high} DC, respectively (12, 25). Depending on culture conditions, a varying degree of the DC may spontaneously express high levels of MHC class II. Using this grouping as well as the levels of B7-2 expression, the isolation and culturing methodology used here was first evaluated by analyzing DC stimulated with LPS as a positive control or 2-μm latex beads as a negative control (Fig. 3). Typically, 20–30% of the unstimulated cells displayed the MHC class II^{high} phenotype (Fig. 3*A*) in the optimized system. Both consistent handling of cells as well as use of appropriate GM-CSF concentration during the culture and stimulation period were found to be factors of utmost importance in minimizing the number of DC spontaneously expressing high levels of MHC class II. By stimulation with LPS (1 μg/ml), the portion of MHC class II^{high} cells increased to 50–55%. The range of cells expressing high levels of B7-2 in unstimulated and LPS-stimulated cultures were 20–25% and 50–60%, respectively. Latex beads at concentrations comparable to the concentration levels of bacteria applied in this study (10^6 and 10^7 particles/ml) were ineffective in up-regulating MHC class II and B7-2 (Fig. 3*B*). Microscopic examination of the DC exposed to latex beads clearly revealed that phagocytosis of the particles had taken place after 12 h of incubation. Thus, phagocytosis of the lethally irradiated bacterial particles alone was not sufficient for up-regulation of MHC class II or B7-2 expression.

The capacity for the various *Lactobacillus* spp. to induce up-regulation of MHC class II and B7-2 was tested at different concentrations of whole, washed, irradiated bacteria (1, 10, and 100 μg/ml). The greatest difference among the six species analyzed regarding both MHC class II and B7-2 expression was observed between *L. reuteri* and *L. casei*, whereas the remaining four species showed similar patterns. Flow cytometry data for MHC class II and B7-2 are displayed in Figs. 4 and 5, respectively, which, in addition to results for *L. reuteri* and *L. casei*, include data obtained for *L. johnsonii* La1 representing typical results for *L. plantarum* Lb1, *L. plantarum* 299v, and *L. fermentum* Lb20.

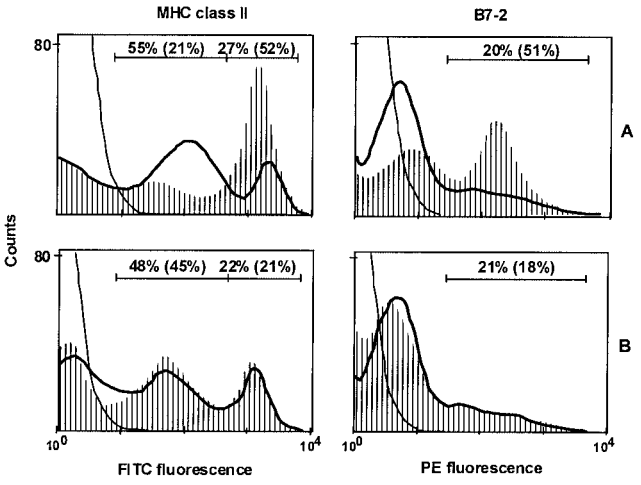

FIGURE 3. Expression of MHC class II and B7-2 is up-regulated by LPS but not by latex particles. Histograms represent flow cytometry results from 8-day old bone marrow-derived DC cultures stimulated for an additional 12 h with either LPS (1 μg/ml; *A*) or 2-μM latex beads (10^7 beads/ml; *B*) (filled histograms) in comparison with unstimulated cells (thick solid line). Numbers above peak indicate the percentage of unstimulated cells falling in the range of the marker, whereas percentages in parentheses relate to stimulated cell values. Curves are normalized to the values for unstimulated cells. Isotype control is shown by a thin solid line. Data are representative of four independent experiments.

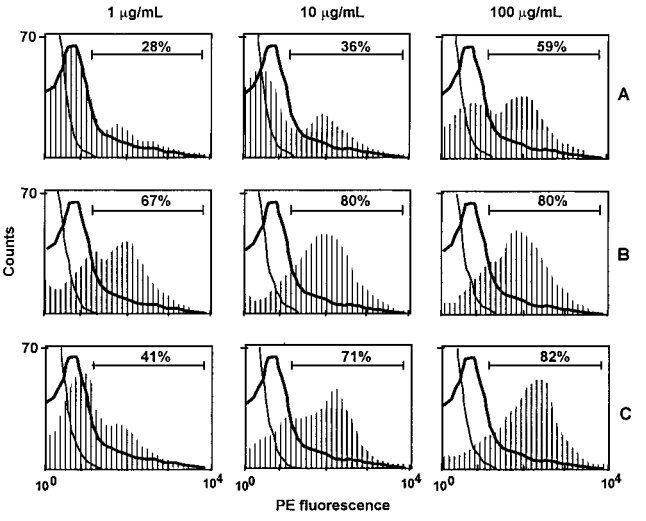

FIGURE 5. *Lactobacillus* spp. differentially up-regulate B7-2 expression on DC. Histograms show flow cytometry results of B7-2 expression on bone marrow-derived DC cultures stimulated with different species of *Lactobacillus* (*A, L. reuteri*; *B, L. casei*; and *C, L. johnsonii* La1) at various concentrations (filled histograms) in comparison with unstimulated DC (thick solid line). Numbers above peak indicate the percentage of cells falling in the range of the marker. Curves are normalized to the values for unstimulated cells. Isotype control is shown by a thin solid line. The data are representative of two independent experiments.

In general, *L. casei* showed a stronger capacity to up-regulate MHC class II than the other species of *Lactobacillus*, whereas *L. reuteri* showed the weakest capacity (Fig. 4). Even at a concentration of 10 μg/ml, *L. reuteri* failed to induce MHC class II expression to the same extent as *L. casei* at concentrations as low as 1 μg/ml when using the percentage of MHC class II^{high} DC as well as the total percentage of MHC class II^{low} and MHC class II^{high} DC.

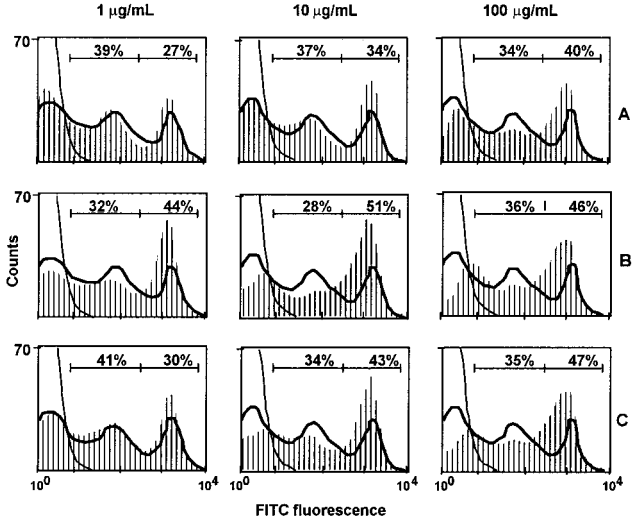

FIGURE 4. *Lactobacillus* spp. differentially up-regulate MHC class II expression on DC. Histograms show flow cytometry results for MHC class II expression on bone marrow-derived DC cultures stimulated with different species of *Lactobacillus* (*A, L. reuteri*; *B, L. casei*; and *C, L. johnsonii* La1) at various concentrations (filled histograms) in comparison with unstimulated DC (thick solid line). Numbers above peak indicate the percentage of cells falling in the range of the marker. Curves are normalized to the values for unstimulated cells. Isotype control is shown by a thin solid line. The data are representative of two independent experiments.

Similar but more marked trends were found when analyzing for B7-2 up-regulation (Fig. 5). While *L. reuteri* at 1 μg/ml failed to up-regulate B7-2 expression, *L. casei* at the same concentration strongly induced B7-2 expression. The effect of *L. casei* at 1 μg/ml on B7-2 expression was even greater than that induced by 10 μg/ml *L. reuteri*. Notably, B7-2 expression on DC was up-regulated by the lactobacilli to a much higher level than that induced by an optimized concentration of LPS (~80 vs ~55%, respectively; Fig. 3).

L. reuteri *inhibits the* L. casei*-induced up-regulation of B7-2*

Because *L. reuteri* had only weak capabilities relative to *L. casei* to up-regulate DC MHC class II and B7-2 and, furthermore, could inhibit *L. casei*-induced cytokine production, the effect of combining these two bacteria on surface marker expression was assessed. DC were exposed to a fixed concentration of *L. casei* of 1 μg/ml in combination with varying concentrations of *L. reuteri* ranging from 0 to 30 μg/ml (Fig. 6). In preliminary experiments, a concentration of *L. reuteri* of ~30 μg/ml was found approximately as effective as 1 μg/ml *L. casei* in inducing the surface markers. Based on the portion of DC expressing a surface marker intensity in the range indicated by a marker (Fig. 6, *A* and *C*), the effects of increasing amounts of *L. reuteri* on DC cultured with and without *L. casei* were compared (Fig. 6, *B* and *D*). These graphs reveal that *L. reuteri* caused a dose-dependent but modest reduction of *L. casei*-induced MHC class II expression (Fig. 6*B*). In comparison, expression of B7-2 was more strongly inhibited and also followed a dose-dependent pattern (Fig. 6*D*). For both MHC class II and B7-2, the maximal inhibition by *L. reuteri* reduced the level of expression down to the level induced by *L. reuteri* alone, indicating a complete abolition of the *L. casei* effect by *L. reuteri*.

Discussion

Numerous investigations have been conducted on the effect of probiotic bacteria including the lactobacilli on immune function. Interpretation of the resultant findings has, in general, tended to be

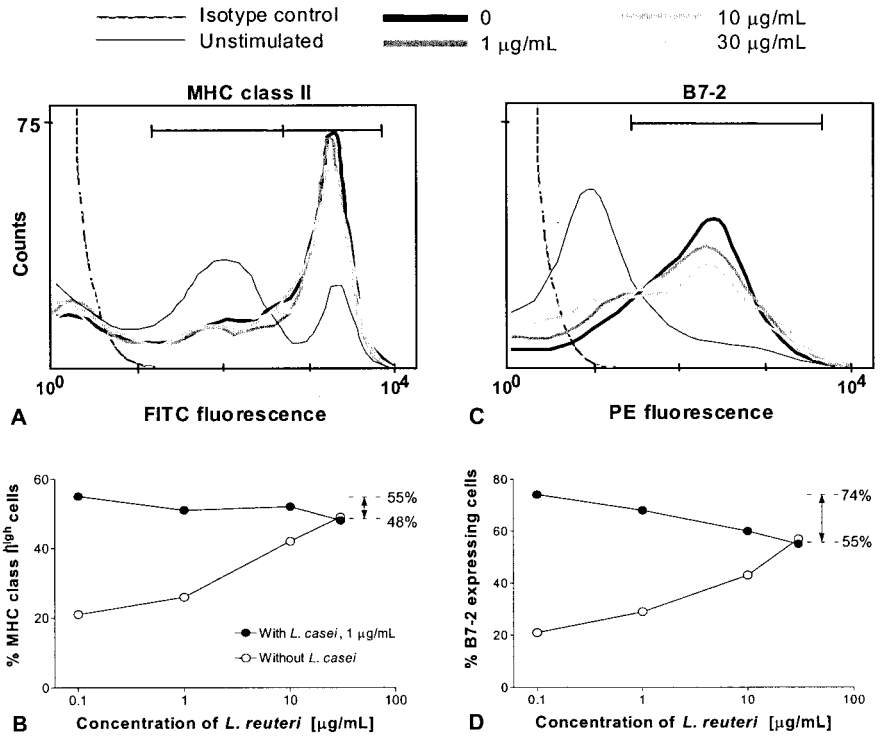

FIGURE 6. *L. reuteri* inhibits *L. casei*-induced expression of MHC class II and B7-2 on DC. All bone marrow-derived DC cultures were stimulated with *L. casei* (1 μg/ml) in combination with variable concentrations of *L. reuteri* (0–30 μg/ml). Overlays of flow cytometry histograms for MHC class II (*A*) and B7-2 (*C*) prepared at various concentrations of *L. reuteri* in combination with *L. casei.* Curves are normalized to the values for unstimulated cells. Isotype control is shown by a thin solid line. Flow cytometry was depicted graphically as the percentage of cells expressing a high level of MHC class II (*B*) and B7-2 (*D*) vs concentration of *L. reuteri*. The data are representative of two independent experiments.

inconclusive and even conflicting in the absence of clear mechanistic data. The intestine provides a unique environment for the development of both immunity and tolerance at the same time. There is increasing evidence that the type of initiated immune response relies on the highly polarized functioning of the DC and that this ultimately depends on DC type and state of activation. DC function is greatly influenced by locally present environmental factors (14, 24). DC polarization apparently involves variable expression of both antigenic and costimulatory signals as well as variations in expression of cytokines such as IL-10 and IL-12. Because lactobacilli and other gut-associated bacteria appear to affect immunity at the innate as well as specific humoral and cellular levels, it seemed plausible that DC may play a central role in mediating effects of probiotic bacteria. Moreover, very recent data have shown that DC penetrate the epithelium without disrupting the barrier function and directly sample gut-associated bacteria (28). The data reported herein suggest for the first time that lactobacilli may differentially affect DC maturation as determined by cytokine and surface marker expression.

The capacity of the lactobacilli to variably induce IL-12 and TNF-α, and, to a lesser extent, IL-6 and IL-10, indicates that species of *Lactobacillus* may differentially determine whether a DC favors Th1, Th2, or Th3 immune response. IL-12 and IL-10 produced by APCs exert largely opposite immunoregulatory effects. IL-12 is a critical Th1-skewing cytokine that elicits IFN-γ production by T cells and by NK cells (19). IL-10, on the contrary, is an anti-inflammatory cytokine that suppresses IL-12 production and consequently IFN-γ production, thus favoring a Th2 or Th3 response. Furthermore, IL-10 down-regulates Ag presentation by blocking translocation of Ag-MHC class II complex (29). IL-10 is produced locally in the intestine by a variety of cells such as APCs and mast cells. IL-10 acts on macrophages to prevent their activation and elaboration of proinflammatory molecules and chemokines, thus inhibiting T cell recruitment into the intestine (30).

The potent capacity of *L. casei* to induce IL-12 is consistent with previous work, in which this strain or the related *L. paracasei* were found to induce high amounts of IL-12 in serum, splenocytes, or blood-derived monocytes (31–33). Using blood-derived monocytes, Hessle et al. (34) found that Gram-positive bacteria are, in general, more potent IL-12 inducers than Gram-negative bacteria. The results obtained herein for *L. reuteri* demonstrate that this bacterial strain deviates from this tendency.

A concentration-dependent cytokine pattern was observed in this study. Specifically, low concentrations of *L. casei* yielded maximal levels of IL-6, IL-12, and TNF-α but virtually no IL-10 induction. At higher concentrations, IL-10 production increased radically with no change in the level of the other cytokines. This observation indicates that the threshold for the bacterial concentration necessary to induce cytokine production varies among cytokines. Accordingly, individual variations in the concentration of bacteria may, in addition to the presence of different types of bacteria, generate different outcomes following interaction with local DC and thus ultimately dictate whether a Th1, Th2, or Th3 response occurs.

IL-6 is produced by a wide spectrum of cells; however, APCs represent the major source. IL-6 promotes terminal differentiation of B cells into plasma cells and has furthermore been found to be able to polarize naive $CD4^+$ T cells to effector Th2 cells (35). The relative strong capacity to induce IL-6 observed for all the *Lactobacillus* strains is consistent with the general finding that many species of lactic acid bacteria induce IL-6 and enhance intestinal IgA responses (36, 37). As with IL-12, *L. reuteri* could down-regulate IL-6 expression and perhaps might attenuate IgA responses.

DC inhabiting the gut mucosa are mostly immature and thus potentially prone to modulation by the environment, which contains an abundance of microorganisms. It has been postulated that intestinal DC expressing a default level of B7-2 produce little IL-12 and thus favor induction of a type 3 response (oral tolerance), whereas active immunity is induced by stimulated DC, which express high levels of B7-2 with either low production of IL-12 (leading to a type 2 response) or high-level IL-12 production (favoring a type 1 response) (14, 20). Homeostasis within this

system is important for the outcome of an immune response to Ags encountered by the mucosal immune system.

The great differences observed here between *L. reuteri* and *L. casei* in the capacity to induce not only production of key cytokines such as IL-12 and IL-10 but also maturation surface markers, especially B7-2, indicate these bacteria may differentially alter Ag presentation in the gut and thus differentially affect the steady state level of DC activation. In possessing a capacity to moderately up-regulate MHC class II and B7-2 on DC, and to induce IL-10 but not IL-12 production, *L. reuteri*, when present in the gut, may contribute to a type 2- or 3-promoting polarization of the DC as opposed to *L. casei*, which in its position as a strong inducer of both surface molecules and IL-12 may promote a type 1 DC polarization. The apparent type 1-promoting capacity of *L. casei* supports the findings that *Lactobacillus* spp. have been found to be antiallergic and to inhibit IgE production (31, 38).

Previously identified DC-polarizing factors include, among others, LPS and bacterial DNA, which favor a type 1 response because they stimulate IL-12 production in DC (23, 39, 40). Potent type 2 DC-polarizing factors, i.e., factors causing IL-12 suppression, include compounds with a cAMP-elevating property such as PGE_2, β_2 agonists, histamine, and cholera toxin (24, 41, 42). IL-10 has been suggested to be a type 3 factor, because it not only suppresses IL-12 but also attenuates the stimulatory capacity of the DC. As type 2 DC-polarizing factors share the property of being IL-12 suppressors, we wanted to test whether this characteristic applied for the poor IL-12 inducer *L. reuteri*. When adding combinations of lactobacilli to DC cultures, *L. reuteri* inhibited IL-12 as well as TNF-α and IL-6 production induced by the otherwise strong cytokine inducer *L. casei*. However, IL-10 was not inhibited by *L. reuteri*, nor was there an effect on cytokines induced by LPS. Similarly, up-regulation of B7-2 caused by *L. casei* was reduced in the presence of *L. reuteri* to the level induced by *L. reuteri* alone. These results suggest that *L. reuteri* may have a DC-polarizing capacity distinct from other lactobacilli strains included in the study and that different species of *Lactobacillus*, in general, may be capable of inhibiting the activities exerted by other bacteria. As IL-10 production is not reduced along with the inhibited IL-12 production, bacteria-induced IL-10 may not derive solely from negative feedback resulting from abundant IL-12 production. These findings suggest that *L. reuteri* may contribute to an environmental modulation of the intestinal DC generation toward favoring tolerance toward Ags bearing no "danger signal" while, at the same time, keeping intact the capacity to respond against pathogens recognized via a danger signal like LPS.

DC recognition of and response to molecular structures on bacteria (pathogen-associated molecular patterns) occurs through a family of pattern recognition receptors designated Toll-like receptors (TLRs). Activation of these surface receptors by bacterial components are believed to be a key factor for regulation of the immune response and to mediate a link between the innate and the adaptive immune function (43). Differential activation of TLR by multitype pathogen-associated molecular patterns could induce different cytokine patterns. This fact might explain not only the observed differences in cytokine induction found by the *Lactobacillus* included in this study but also the divergent results obtained from studies on immunological effects of the gut flora. TLR2 has been found to be a signal transducer for cell activation by peptidoglycan, lipoteichoic acid, and bacterial lipoprotein in addition to LPS (44). However, as LPS also binds to TLR4 and CD14, these receptors constitute alternative activation pathways for LPS (45). Therefore, it could be speculated that the inhibiting effect of *L. reuteri* is mediated through interference with receptors like the TLR2, causing a rather universal inhibition toward other lactobacilli and possibly Gram-positive bacteria but leaving the LPS stimulation through CD14 unaffected. Alternatively, other TLR(s) activated by the bacteria may be involved. Notably, no isolated bacterial component has been found entirely responsible for the potent induction of IL-12 seen for whole bacteria, indicating that multiple receptors may be involved in mechanisms of stimulation (34). The involvement of TLR in the differential activation capacities of species of *Lactobacillus* and other bacteria on DC provides an intriguing target for future mechanistic studies.

The results presented in this work have potential importance from a clinical standpoint. Colonization of the neonatal gut by bacteria with properties resembling those found here for *L. casei* may be an important type 1 driving factor for the Th2-skewed immature immune system to establish a Th1/Th2 balance optimal for coping with encountered dietary Ags, particularly for atopic individuals more prone to develop allergy. As the postnatal colonization of the gut occurs within 1 wk with microbial species originating from the environment, it is plausible that the type of colonizing species could be modified by administration of bacteria with certain properties (3). Considering the properties observed for *L. reuteri*, which from an immunological point of view are opposite those observed for *L. casei*, bacteria with such properties might be beneficial for patients with inflammatory bowel diseases. The cytokines IL-12 and TNF-α are both implicated in the enteropathy of these diseases, as they are increased in the mucosa of patients suffering from diseases like Crohn's and for whom administration of TNF-α-neutralizing Abs has been a successful treatment (46). Conceivably, bacteria like *L. reuteri* might be a potential fine-targeted treatment effective for down-regulating production of IL-12 and TNF-α while inducing the anti-inflammatory IL-10, thus representing an alternative therapeutic approach to counterbalance the proinflammatory intestinal cytokine milieu.

Taken together, the results of this study suggest that species of *Lactobacillus* exert very different and even opposing effects on DC activation. Because DC are crucial for the induction of such diverse Ag-specific immune responses as oral tolerance and Th1/Th2 immunity, and because bacterial colonization of the gut appears mandatory for development of a competent immune function of the host, the presence of probiotic bacteria or components thereof during the development of the DC may play a role in determining the outcome of a response. It might be speculated that *L. casei*, as a strong IL-12 and TNF-α inducer as well as MHC class II and B7-2 inducer, could be a contributing type 1 DC-polarizing factor when present in appropriate amounts in the gut, whereas *L. reuteri*, due to the DC-stimulating capacity characterized by only a weak MHC class II and B7-2 induction and suppressed IL-12 induction, could be a type 2 or 3 DC-polarizing bacteria. The model described in this work could be adapted in future studies to assess whether DC incubated with the various strains of lactobacilli differ in their ability to activate T cells or to alter T cell phenotype. Improved understanding of the effect of the gut flora, including probiotics, on cells controlling gut immune function is crucial for the precise design of effective bacteria-mediated interventions that seek to modulate potent immunoregulatory mechanisms. As the model system used in the present work involves in vitro exposure of bone marrow-derived DC to the species of *Lactobacillus*, it cannot be directly implemented as a model for testing the effect of these bacteria on the gut-associated DC. Nevertheless, the method clearly illuminated distinct DC activation properties of individual as well as combinations of bacteria and thus has potential as a rapid preclinical screening method. Further studies are needed to elucidate the active bacterial cellular components causing the variable effects observed for *L. reuteri* and *L. casei* as well as extension of the work to an in vivo model.

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