Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization

Mansour Mohamadzadeh*, Scott Olson†, Warren V. Kalina‡, Gordon Ruthel*, Gretchen L. Demmin*, Kelly L. Warfield*, Sina Bavari‡, and Todd R. Klaenhammer†

*National Cancer Institute, Frederick, MD 21702; †Department of Microbiology, Louisiana State University, New Orleans, LA 70112; ‡Center for Gene Therapy, Tulane University, New Orleans, LA 70112; §U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702; and †Department of Food Science, North Carolina State University, Raleigh, NC 27695

Contributed by Todd R. Klaenhammer, January 6, 2005

Professional antigen-presenting dendritic cells (DCs) are critical in regulating T cell immune responses at both systemic and mucosal sites. Many Lactobacillus species are normal members of the human gut microflora and most are regarded as safe when administered as probiotics. Because DCs can naturally or therapeutically encounter lactobacilli, we investigated the effects of several well defined strains, representing three species of Lactobacillus on human myeloid DCs (MDCs) and found that they modulated the phenotype and functions of human MDCs. Lactobacillus-exposed MDCs up-regulated HLA-DR, CD83, CD40, CD80, and CD86 and secreted high levels of IL-12 and IL-18, but not IL-10. IL-12 was sustained in MDCs exposed to all three Lactobacillus species in the presence of LPS from Escherichia coli, whereas LPS-induced IL-10 was greatly inhibited. MDCs activated with lactobacilli clearly skewed CD4+ and CD8+ T cells to Th helper 1 and Tc1 polarization, as evidenced by secretion of IFN-γ, but not IL-4 or IL-13. These results emphasize a potentially important role for lactobacilli in modulating immunological functions of DCs and suggest that certain strains could be particularly advantageous as vaccine adjuvants, by promoting DCs to regulate T cell responses toward Th1 and Tc1 pathways.

Lactobacilli are part of the commensal microbial flora of the intestinal tract of humans and mammals and are generally recognized as nonpathogenic. Dense cultures of viable organisms may be administered by a variety of mucosal routes (1–4). It is well documented that the intestinal microflora contributes to the health of the host with various bacterial species known to modulate immune responses (1–5). The mechanisms of such immune modulations are unknown. However, it has been demonstrated that the cell wall of these bacteria contain immunomodulatory components such as cell surface components and peptidoglycan that may play an important role in activating immune-competent cells in the intestine (3). Furthermore, functional involvement of the intestinal microflora in modulation of immune responses and maintenance of homeostasis highlights the critical role of the microbiota in our intestine (6, 7). Furthermore, intestinal microbiota, which include various species of Lactobacillus, interact regularly with cells of the colon, which include professional antigen-presenting cells and intestinal epithelial cells (8–10). Moreover, it has recently been reported that lactobacilli may facilitate the polarization of the naïve immune system by skewing it away from Th helper 2 (Th2) toward Th1 responses, and thus promoting humoral and cell mediated immunity (11).

Dendritic cells (DCs) play a pivotal role in immunological responses by priming adaptive immunity. Immature DCs migrate through the bloodstream and home to various tissues where they confront invading pathogens. Migratory DCs in the periphery, lymphatic, and nonlymphatic organs then undergo phenotypic and functional changes, including up-regulation of cell surface expression of costimulatory and adhesion molecules and production of inflammatory chemokines and cytokines (12–15). Along with antigen uptake and processing, these functional changes in the DCs augment and direct both humoral and adaptive immune responses (12, 13). Depending on the microbial stimulus encountered, DCs can promote the development of unprimed, naive T cells toward Th1, Th2, or unpolarized T cell responses (12, 13).

The gastrointestinal tract is colonized by an assortment of commensal bacteria, which are the primary stimulus for the intestinal immune system (16). Therefore, DCs residing in several compartments of the gut regularly encounter nonpathogenic organisms of the gut microflora, including Lactobacillus species (15, 16). It has been postulated that Lactobacillus cells may modulate DC properties, including their ability to activate specific immune responses at mucosal sites (14–16). A balance of DC stimulation and tolerance after an encounter with Lactobacillus cells in the gut may be important to maintain the homeostasis required for symbiotic bacteria to perform their critical functions in host nutrition, intestinal permeability, and protection against foreign, pathogenic microbes (16). In this study, we examined DC responses to three Lactobacillus species and investigated whether or not these bacteria could induce T cell immune responses in immature human DCs.

Materials and Methods

Bacterial Strains. Lactobacillus gasseri (ATCC no. 19992), Lactobacillus johnsonii (ATCC no. 33200), and Lactobacillus reuteri (ATCC no. 23272) were obtained from American Type Culture Collection. Lactobacillus species were inoculated at 1% and propagated in de Man, Rogosa, and Sharpe broth (MRS, Difco) at 37°C for 15 h. Subsequently, 1 ml of each culture was then transferred to 500 ml of fresh MRS and incubated at 37°C for 8 h until mid-log phase. Cells were then harvested by centrifugation, washed with PBS (50 ml), and added to immature myeloïd DCs (MDCs). To kill Lactobacillus cells (1011 colony-forming units per ml), the bacteria were exposed to UV-light for 15 min and frozen at −80°C. Complete loss of cell viability was verified by plate counts on MRS medium. The dry cell weight of bacterial concentrations was determined by freeze-drying aliquots and correcting for buffer salt content. LPS from Escherichia coli was purchased from Sigma (SF3–82).

Abs, Cytokines, and Reagents. Murine mAbs were: HLA-DR, CD3, CD4, and CD8 (Becton Dickinson); CD62L (Caltag, South San Francisco, CA); CD83 (Pharmingen); CD40, HLA-ABC (R & D Systems), CD1a (DAKO); CD80, CD45RA, CD45RO, and CD69 (Beckman Coulter, Fullerton, CA). Recombinant human granulocyte/macrophage colony-stimulating factor was purchased from BioSource International (Camarillo, CA). Recombinant human IL-4 was purchased from R & D Systems. All ELISA reagents were purchased either from Pharmingen, R & D Systems, or BioSource International.

Abbreviations: DCs, dendritic cells; Th, T helper; TLR, Toll-like receptor; MDC, myeloid DC.

*To whom correspondence should be addressed. E-mail: mmadeh@nifcrf.gov.

© 2005 by The National Academy of Sciences of the USA
DCs. Peripheral blood mononuclear cells were isolated from the blood of healthy individuals by Ficoll gradient centrifugation. Peripheral blood mononuclear cells including monocytes (107 cells per well) were seeded in six-well plates for 2 h at 37°C. Nonadherent cells were removed by several washes by using PBS plus 2% heat inactivated FCS and frozen for autologous or allogeneic mixed lymphocyte reaction experiments. Adherent monocytes were cultured with human granulocyte/macrophage colony-stimulating factor (100 ng/ml) and human IL-4 (10 ng/ml) in complete medium consisted of RPMI 1640, 10% FCS, 1% l-glutamine, 1% penicillin/streptomycin, 50 μM 2-mercaptoethanol, 1% sodium pyruvate, and 1% nonessential amino acids (all from GIBCO) for 6 d (17, 18). MDCs on day 5 of culture were defined as immature MDCs. Immature MDCs were treated with Lactobacillus cells at various doses: 10:1, 100:1, or 1,000:1 colony-forming units per MDC for various times of 16, 48, or 72 h at 37°C. Treated MDCs were then harvested, centrifuged at 300 × g for 10 min, washed three times with PBS, and then used in several experiments. To detect intracellular IL-10 and IL-12 expression, MDCs were stimulated with lactobacilli or E. coli LPS (100 ng/ml) at 37°C for 2–3 d and treated with golgi-inhibitor for 4 h. MDCs were stained for CD1a or HLA-DR, fixed with 0.1% paraformaldehyde, permeabilized, and stained with IL-10 antigen-presenting cells and IL-12 FITC for 1 h at 4°C. Subsequently, cells were washed and analyzed by flow cytometry. In some experiments, the supernatants of MDCs activated with Lactobacillus species or E. coli LPS (100 ng/ml) were collected and assayed for IL-1β, TNF-α, IL-6, IL-10, IL-18, or IL-12 p70 by ELISA, according to standard protocols.

T Cell Proliferation. Isolated CD4+ T cells were negatively purified by the depletion of CD8+, CD19+, CD56+, CD1a+, and CD14+ cells by using specific Ab conjugated beads (Miltenyi Biotec, Auburn, CA). CD8+ T cells were purified by depleting CD4+ cells in analogous fashion (29). Monocyte-derived DCs were generated as described above and incubated with live or killed Lactobacillus cells, E. coli LPS (300 ng/ml), or with no supplement for 72 h at 37°C. MDCs were harvested and washed with PBS. Subsequently, MDCs were cocultured at graded doses with autologous or allogeneic CD4+ or CD8+ T cells (105 cells per well in a 96-well plate) for 4 d in complete RPMI medium 1640 where heat-inactivated 10% human AB serum (Gemini Bio-Products, Woodland, CA) replaced FCS. Cells were pulsed with 0.5 μCi of [3H]thymidine per well (New England Nuclear). [3H]Thymidine incorporation was measured 16 h later by using a β-counter (Wallac TriLux, Perkin–Elmer). In some experiments naive CD62L+CD45RA+CD45RO−CD4+ or CD62L+CD45RA+CD45RO−CD4+CD8+ T cells were sorted by a cell sorter from human adulate peripheral blood mononuclear cells. Subsequently, autologous or allogeneic CD4 (5 × 105 cells per well in a 96-well plate) or CD8 T cells were cocultured with MDCs, which were treated with live or killed Lactobacillus species as described above. Supernatants of the cocultures were harvested on day 5 and subsequently assayed by ELISA to measure cytokines.

Flow Cytometry. MDCs (5 × 105) were incubated with mAbs or isotype control for 1 h at 4°C, washed extensively with PBS plus 0.2% FCS, fixed with 0.1% paraformaldehyde, and analyzed by a FACSCalibur four-laser cytometry by using standard CELLQUEST acquisition/analysis software (Becton Dickinson). At least 1 × 106 gated events per condition were acquired. FACS analysis for intracellular IL-10 and IL-12 was performed as described (19).

Confocal Microscopy. Untreated or treated MDCs with Lactobacillus species or with E. coli LPS were stained by using anti-HLA-DR Ab and an Alexa Fluor 488 conjugated anti-mouse secondary Ab, Texas red phalloidin, and Hoechst dye (Molecular Probes). Cells were visualized with a Bio-Rad 2000MP confocal multiphoton microscope. Image acquisition and subsequent contrast enhancement were done identically for each condition.

RT-PCR. RT-PCR amplifications were performed as described (20). Cycling conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The primers used to amplify Toll-like receptor 2 (TLR-2) and β-actin genes were TLR-2, (forward) 5’T-TGAGGTTTCGACCTCTGTG3’ and (reverse) 5’GCCAAGTCTTTGATGTATG3’ and β-actin, (forward) 5’CGAGGCCCAACCCGAGAAGA3’ and (reverse) 5’-GTCGCCGCGACCAGGTCCCA-3’. Results

Lactobacillus Species Modulate the Phenotype and Function of MDCs. Previous studies have shown that Lactobacillus plantarum and Lactobacillus rhamnosus can induce activation, as measured by
Lactobacillus Species Induce Bioactive IL-12 and IL-18 and Proinflammatory Cytokines in MDCs. The production of chemokines and cytokines is another critical function of DCs in response to microbial stimulation (12). Therefore, the production of the proinflammatory cytokines IL-12 (Fig. 2 A–C) and IL-18 (Fig. 2D), as well as the antiinflammatory cytokine IL-10 (Fig. 2A–C) were examined. Killed (data not shown) or live Lactobacillus-treated MDCs produced bioactive IL-12 p70, but not IL-10, as determined by ELISA (Fig. 2A). This observation was confirmed by intracellular staining (Fig. 2B). In contrast, MDCs stimulated with E. coli LPS secreted high levels of IL-12 p70, IL-10, and IL-18, as expected (Fig. 2A–D). To investigate whether Lactobacillus activation inhibited LPS-mediated IL-10 induction, MDCs were activated with live or killed (data not shown) L. gasseri, L. johnsonii (data not shown), or L. reuteri plus E. coli LPS for 2 d. Under these conditions, LPS promoted the production of IL-10 only in 11% of MDCs, compared with the high (60%) induction of IL-10 with E. coli LPS in the absence of L. reuteri (Fig. 2 B and C). Lethally-irradiated Lactobacillus species (10 µg/ml) also activated MDCs to secrete IL-12 p70 and IL-18, but not IL-10, respectively (data not shown). Moreover, IL-6 was induced in MDCs at similar levels when treated with each Lactobacillus species or E. coli LPS (Fig. 2E). IL-1β was minimally induced in MDCs treated with L. johnsonii, L. gasseri, or E. coli LPS but was induced in MDCs treated with L. reuteri (Fig. 2F). TNF-α production was observed in MDCs treated with Lactobacillus species or E. coli LPS (Fig. 2G). To test whether L. johnsonii can inhibit the elevated production of IL-1β induced by L. reuteri, DCs were treated with live or killed (data not shown) L. johnsonii and L. reuteri. Data showed that L. johnsonii did not inhibit the high secretion of IL-1β induced by L. reuteri in DCs (Fig. 2H).

Induction of T Cell Proliferation and Activation by MDCs Treated with Lactobacillus Species. DCs affect the adaptive immune response by priming T cells to proliferate, become activated, and

Fig. 2. Induction of bioactive IL-12, IL-18, and proinflammatory cytokines in MDCs treated with Lactobacillus cells. MDCs were treated with Lactobacillus species, E. coli LPS, or no supplement for 3 d at 37°C. (A) Supernatants of MDCs treated with Lactobacillus species, E. coli LPS, or no supplement were harvested after 72 h and analyzed for IL-10 and IL-12 by ELISA. Experiments were performed at least three times with similar results. (B) MDCs were treated with Lactobacillus species, E. coli LPS, L. reuteri and E. coli LPS, or no supplement for 48 h. MDCs were harvested and treated with Golgi inhibitor for 4 h. Cells were stained with anti-IL-10 allophycocyanin or anti-IL-12 FITC for 1 h on ice. Cells were washed and analyzed by flow cytometry. (C) DCs were treated with LPS alone or in combination with Lactobacillus species for 72 h. Cytokines were then analyzed by ELISA. (D) Supernatants of MDCs treated with Lactobacillus species, E. coli LPS, or no supplement were harvested after 72 h and analyzed for IL-18 by ELISA. Experiments were performed at least three times with similar results. (E–H) MDCs supernatants were harvested and analyzed for proinflammatory cytokines by ELISA. Results are representative of three independent experiments.
produce cytokines (12). When MDCs were activated with live (Fig. 3A and B) or killed (data not shown) lactobacilli, they enhanced the proliferation of allogeneic CD4⁺ and CD8⁺ T cells, confirming their capacity to enhance priming of T cells. As expected, MDCs activated with E. coli LPS increased CD4⁺ T cell proliferation and enhanced the proliferation of allogeneic CD8⁺ T cells (Fig. 3A and B). Interestingly, MDCs activated with live (Fig. 3C and D) or killed (data not shown) lactobacilli strongly induced the proliferation of autologous CD4⁺ and CD8⁺ T cells. By contrast, no T cell proliferation was observed in autologous CD4⁺ or CD8⁺ T cells when cocultured with MDCs activated with E. coli LPS (Fig. 3C and D). MDCs activated with live (Fig. 4A) or killed (data not shown) lactobacilli induced high levels of IFN-γ in both allogeneic T cells; however, IL-10 production was observed in autologeneic CD4⁺ with only minimal induction of IL-10 in CD8⁺ T cells (Fig. 4B). When MDCs were treated with various Lactobacillus species, the MDCs induced high levels of IFN-γ but minimal or no IL-4, IL-10, or IL-13 in autologous CD4⁺ or CD8⁺ T cells (Fig. 4C and D and data not shown). Furthermore, in an attempt to show whether activated MDCs with lactobacilli can enhance the proliferation and activation of naïve CD4⁺ or CD8⁺ T cells, MDCs were treated with live or killed (data not shown) cells and then were cocultured with naïve allogeneic or autologous CD4⁺ and CD8⁺ T cells. Activated MDCs with lactobacilli enhanced allogeneic T cell proliferation (Fig. 5A and B) and induced the secretion of IFN-γ and IL-2, but not IL-4 or IL-13 by naïve CD4⁺CD62L⁻CD45RA⁺CD45RO⁻ or CD8⁺CD62L⁻CD45RA⁺CD45RO⁻ T cells (Fig. 5C and D). Mild enhancement of IL-10 was observed, similar to that shown previously in Fig. 4A and B. In contrast, no proliferation or activation of naïve T cells was induced by autologous MDCs treated with live or killed (data not shown) lactobacilli. These results may indicate that Lactobacillus-activated MDCs induce the activation of effector memory T cells specific for bacterial components.

**Up-Regulation of TLR-2 in MDCs by Lactobacilli.** Because MDCs were efficiently activated, we investigated whether TLR-2 was induced after DC engagement of Lactobacillus cells. Expression of
TLR-2 was undetectable on MDC surface by FACS analysis (data not shown). Therefore, RT-PCR analysis was performed on activated MDCs by using primers that were specific for RNA transcripts encoding either TLR-2 or β-actin. MDCs activated with lactobacilli or E. coli LPS showed a 3-fold induction of TLR-2 transcripts whereas β-actin expression remained constant, as expected (Fig. 6).

Discussion

The commensal microbial flora of the intestinal tract harbors Gram-positive and Gram-negative bacteria that may be involved in homeostasis of gut-associated immunity (3–5, 16). Recent studies have highlighted the effects of probiotic bacteria on immune competent cells (3, 4, 24–26). In the present study, we investigated the adjuvanticity of three Lactobacillus species on immature human MDCs and showed that Lactobacillus cells induced activation and maturation of MDCs. Furthermore, we found that Lactobacillus-exposed MDCs secreted bioactive IL-12, a critical factor in switching naive or memory T cells to Th1 responses, which are proinflammatory and lead to robust immunity against infections and other diseases (27).

Interestingly, IL-12 production by MDCs induced by lactobacilli was not reversible when MDCs were simultaneously treated with E. coli LPS. This finding suggests that some strains may possess a property that establishes a continuous Th1 immune response by inducing bioactive IL-12 production but not IL-10 production. In agreement with these results, it has been shown that macrophages treated with lactobacilli activated NFκB and STAT signaling, resulting in secretion of IL-12 and IL-18 (28). In addition, some Lactobacillus species can differentially effect antigen-specific IgG1 and IgG2Ab responses (29, 30), and inhibit Th2 cytokines (IL-4 and IL-5) derived from patients allergic to house dust mites (31). Our findings show both similarities and differences to previous studies with Lactobacillus and human MDCs (21–23). Similar to our current work, the previous studies found that human MDCs exposed to lactobacilli increased MHC, costimulatory, adhesion, and activation molecules (21–23). However, coculture of MDCs for 24 h with L. rhamnosus and L. plantarum did not elicit IL-2, IL-6, IL-8, or IL-12p70 (21–23, 32). Furthermore, work with murine monocyte-derived DCs by Drakes et al. (33) showed that probiotics containing lactobacilli could induce cell surface markers of maturation and activation, but the DCs produced IL-10 and not IL-12p70 (33). Another study by using mouse bone marrow-derived DCs found an induction of Th2 immune responses when DCs were treated with L. reuteri (34). In contrast, human MDCs treated with L. reuteri in the present study induced Th1 polarization. MDCs treated with L. reuteri or L. johnsonii induced the production of both IFN-γ and IL-10 in allogeneic CD4+ T cells, but IL-10 was not induced in CD8+ T cells. In contrast, L. gasseri induced a clear Th1 polarization pattern in both allogeneic CD4+ and CD8+ T cells. Together, these data suggest that lactobacilli can exert different effects on human immune cells, when compared with mouse immune cells. These differences with human MDCs may be due to several reasons, including differences in the Lactobacillus species, specific strains used, and the timing of sampling to assay cytokine production. IL-10 is thought to be a key for maintaining gut homeostasis and the antiinflammatory effects of probiotics (16). However, we found that the three Lactobacillus species induced IL-12p70, but not IL-10, in MDCs. These observations are further supported by the fact that MDCs activated with lactobacilli primed allogeneic CD4+ and CD8+ T cells and skewed them toward a Th1 response by secretion of IFN-γ. Lactobacilli were far more potent in priming CD8+ T cells compared to E. coli LPS, which is likely due to the production of IL-12p70 and not IL-10 by Lactobacillus-exposed MDCs (35). All three species of lactobacilli clearly stimulated Th1 polarization in allogeneic CD8+ T cells. L. gasseri induced
high levels of IFN-γ, but a low level of IL-10. In contrast, MDCs treated with L. reuteri or L. johnsonii induced IL-10 in allogeneic CD4+ T cells. These species-specific effects are in accordance with previous observations that lactobacilli control the secretion of critical immune modulators, including IFN type I and II, IL-12, or IL-18 in a strain-dependent manner (28). The mechanisms underlying the different MDC responses induced by varying Lactobacillus species and strains are unknown.

Interestingly, Lactobacillus-activated MDCs also induced proliferation of autologous CD4+ and CD8+ T cells and induced their secretion of IFN-γ. The mechanism for this unusual property remains to be defined. However, for autologous naive CD4+ or CD8+ T cells, Lactobacillus-activated MDCs did not induce their proliferation or activation. These results may represent a recall response to endogenous lactobacilli or polyclonal T cell activation, as demonstrated previously for Toxoplasma gondii and in Chlamydia trachomatis infection (35–37).

The findings presented herein add to the complexity of current evidence indicating that intestinal bacteria and probiotics, including lactobacilli, help maintain gut homeostasis by balancing proinflammatory and antiinflammatory mucosal responses (13). We have shown that MDCs respond to certain lactobacilli with inflammatory cytokines that lead to the development of Th1 immune responses. Likely, this represents one piece of a larger, complex environment in which the responses of multiple immune cells, in combination with the responses of other critical intestinal cell types, such as epithelial cells, are responsible for the overall response to nonpathogenic commensal bacteria (16). The responses of the epithelium and other intestinal cells may either combine with or direct the DC response to the microbiota of the gut. In this way, the gut may maintain a balance between inflammatory responses to pathogens and natural intestinal homeostasis and function.

It has been shown that the two major bacterial cell wall components, peptidoglycan in the case of Gram-positive bacteria, and LPS in Gram-negative bacteria, are important molecular markers recognized by the immune system (38). Cell surface molecules such as TLRs and CD14 interact with peptidoglycan or LPS to control expression of several specific, inducible immune responses (38). Accordingly, TLR-2 has been shown to be a signal transducer for cells activated by peptidoglycan, lipoteichoic acid, bacterial lipoprotein, and LPS (39). The Lactobacillus species used in our studies, like LPS, up-regulated expression of TLR-2 transcripts. These data suggest that lactobacilli may deliver signals in MDCs through TLR-2, thereby promoting the activation of these cells.

In summary, three Lactobacillus species were able to activate MDCs to induce strong Th1 cell immunoreactions. Both autologous and nonautologous T cell priming, as well as autologous T cell activation, was detected. This study indicates that various lactobacilli can be efficient immune modulators, but the signals for directing Th1 or Th2 responses are unknown, and appear to vary among strains and species. Because lactobacilli can activate MDCs, prime T cells, and induce Th1 cytokines, certain strains and species could be particularly useful for delivery of biotherapeutics and vaccines. This field is rapidly expanding as the potential for use of recombinant lactic acid bacteria in human health is being recognized and exploited (40).

We thank Dr. Alan Schmaljohn (U.S. Army Medical Research Institute of Infectious Diseases) for support and fruitful discussions and Kristian Finstad and Haleh Mirafab for excellent technical assistance. This work was supported by National Institutes of Health Grant AI 59590-01 and National Institutes of Health–Tulane Base Grant RR00164 (to M.M.).