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Assessment of *Lactobacillus gasseri* as a Candidate Oral Vaccine Vector

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*Lactobacillus* species are commensal bacteria that have long been recognized as probiotic microbes and are generally regarded as safe (GRAS) for human consumption. We have investigated the use of *L. gasseri* as a vaccine vector for oral immunization against mucosal pathogens. Recent research has shown that the immune response to different lactobacilli can vary widely depending on the species or subspecies of *Lactobacillus* being studied. While some lactobacilli seem to induce oral tolerance, others induce an adaptive immune response. This study characterized the systemic and mucosal immune response to wild-type and genetically modified *L. gasseri*. *L. gasseri* primarily activates TLR2/6, with additional activation through the TLR2 homodimer. To expand the Toll-like receptor (TLR) activation profile of *L. gasseri* and the immunogenicity of the vector, a plasmid containing *fliC*, the gene encoding bacterial flagellin, was introduced which resulted in the strong activation of TLR5. The treatment of human myeloid dendritic cells with recombinant lactobacilli expressing flagellin triggered phenotypic maturation and the release of proinflammatory cytokines. In contrast, bacterial treatment also resulted in a statistically significant increase in IL-10 production. In vivo studies established that treatment with *L. gasseri* led to a diversification of B-cell populations in the lamina propria of the murine colon. Furthermore, treatment with genetically modified *L. gasseri* led to a significant decrease in the percentage of FoxP3+ colonic lymphocytes. Taken together, these data clarify the interaction of *L. gasseri* with the host immune system and support further investigation of the in vivo immunogenicity of *L. gasseri* expressing both flagellin and candidate vaccine antigens.

Several species of lactobacilli have been investigated as antigen delivery vehicles against a spectrum of infectious agents, including *Helicobacter pylori*, tetanus, enterotoxigenic *Escherichia coli*, severe acute respiratory syndrome (SARS)-associated coronavirus, rotavirus, *Brucella abortis*, and human papillomavirus (reviewed in reference 34). Despite the growing evidence that lactobacilli are useful as vaccine vectors (21), it is increasingly clear that different species and even subspecies of lactobacilli interact distinctly with the host immune system (1, 32). Several studies have analyzed the abilities of various *Lactobacillus* species to induce dendritic cell (DC) maturation and cytokine production, showing a range of outcomes that appear to favor tolerance on one end of the spectrum and immune activation on the other (7, 22). There also are clear differences in the ability of lactobacilli species to survive the hostile environment of the upper gastrointestinal (GI) tract and colonize the lower GI tract. Functional genomic studies have greatly improved our understanding of some of these characteristics, but much less is known about the molecular and genetic basis of host immunomodulation by *Lactobacillus* species. One strategy to develop lactobacilli as vaccine vectors is to screen many species and strains using *in vitro* and *in vivo* techniques to identify the best strain for a particular application. The alternative is to fully characterize one promising candidate and then genetically modify that candidate for a particular application. *Lactobacillus gasseri* is a commensal, lactic acid-producing bacterium of the human intestinal and genital tracts and is a GRAS (generally regarded as safe)-status organism. The functional genomics of *L. gasseri* are well characterized, and there is a full complement of tools available for genetic manipulation. A recent study has demonstrated the promise of *L. gasseri* as a vaccine vector, whereby mice were fully protected against challenge with anthrax after immunization with *L. gasseri* expressing the anthrax protective antigen (21). Given its niche in the human microbiome, well-studied genome, and success in preliminary vaccine studies, *L. gasseri* is an attractive candidate for the rational design of an oral vaccine vector. To design a vaccine vector of optimal effectiveness and safety, however, interactions between *L. gasseri* and the host immune system must be clarified.

In the present studies we investigated, in greater depth, both the *in vitro* immunogenic potential of wild-type (WT) and recombinant *L. gasseri* with regard to Toll-like receptor (TLR) activation, dendritic cell activation, and cytokine induction and the *in vivo* immunogenic potential with regard to effector cell modulation in the colonic lamina propria. Also, genetically modified *L. gasseri* that expressed intracellular flagellin was evaluated as a means to expand the TLR activation profile of the vector. Importantly, we investigated whether or not genetic modification adversely affects the bacterium-host interaction in terms of persistence, colonization, and anti-lactobacillus immune responses.

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**TABLE 1. Bacterial strains and plasmids used in this study**

| Strain or plasmid | Genotype or characteristic(s) | Reference or source |
|-------------------|-------------------------------|---------------------|
| pTRK563           | Em; Δcat derivative of pGK12 with lacZ from pBluescript II KS(+) | 4                   |
| pTRK685           | Em; derivative of pORI28 with 777-bp internal fragment of gusA | 4                   |
| pFliCP            | Em; pTRK563 with insertion of PGM promoter and βlC from S. Typhimurium | This study           |
| L. gasseri strains |                               |                     |
| ATCC 33323        | Human origin                  | T. Klaenhammer      |
| ADH               | Human intestinal isolate      |                     |
| NCK 1423          | Em; ADH gusA:pTRK685          | 4                   |
| FliCP             | Em; ATCC 33323 carrying pFliCP| 4                   |
| NCK 1785          | Em; ATCC 33323 carrying pTRK563| This study          |

*NC, culture collection at North Carolina State University, Raleigh, NC.

**DC isolation and coculture.** Untested human blood Buffy coats (n = 5) were obtained from the American Red Cross (Durham, NC) for the isolation of myeloid dendritic cells (mDCs). Buffy coats were centrifuged and the plasma removed. Cells were underlayered with Ficoll-Paque plus (GE Healthcare, Piscataway, NJ) and centrifuged to allow for lymphocyte isolation. The marker-assisted congenic screening (MACS) CD1c+ (BDCA-1) dendritic cell isolation kit (Miltenyi Biotec, Auburn, CA) then was used according to the manufacturer’s instructions to enrich dendritic cells. Purity was consistently greater than 90%.

Enriched CD1c+ DCs were plated in 96-well plates (round bottom, polypropylene) at a concentration of 175,000 DCs per 200 μl of filtered human peripheral blood mononuclear cell (hPBMC) medium (RPMI 1640 with 10% fetal bovine serum [FBS], 1% sodium pyruvate, 1% Glutamax [Invitrogen]). DCs were cultured in duplicate with either 10% MRS-PBS, 50 mM FSL-1 (InvivoGen), or 30 ng/ml rFlt3L (InvivoGen) or as a bacterial coculture with ATCC 33323, NCK 1785, or FliC at 10 bacteria per DC.

Wild-type lactobacilli were grown in MRS broth, and recombinant bacteria were grown in MRS with erythromycin (5 μg/ml). Bacteria were centrifuged, washed with PBS, suspended in hPBMC medium, and added to the DC culture. Following 24 h of incubation, DCs were centrifuged and removed for antibody staining with anti-CD80-fluorescein isothiocyanate (FITC) (clone 2D10; Bio-Legend), anti-CD86-phycocerythrin (PE) (clone IT2.2; Bio-Legend), and anti-human HLA-DR-allophycocyanin (APC) (clone TU36; BD Pharmingen), while supernatants were retained at −80°C for cytokine analysis.

**Cytokine multiplex assay.** DC-lactobacillus coculture supernatants (n = 3 to 6) were analyzed for the presence of IL-2, IL-4, IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), transforming growth factor alpha (TGF-α), IL-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-13, IL-17a, IL-23, IL-24, IP-10, IFN-γ, RANTES, and vascular endothelial growth factor (VEGF) on a Bio-Plex array reader (Luminex xMAP Technology) with Bio-Plex Manager software, version 4.0 (Bio-Rad). Concentrations were calculated from relative intensities of reporter tags using five-parameter logistic regression algorithms to fit the sample data to the standard curves generated in Prism 5 (GraphPad Software, La Jolla, CA). Standards for each cytokine were detectable within the range (lower to upper limits) of 0.1 to 3.6 and 432 to 4,600 pg/ml, and only sample data falling within those ranges were used for statistical analysis.

**Mouse inoculation.** Mice were housed and cared for in accordance with Association for the Assessment of Laboratory Animal Care Standards and Institutional Animal Care and Use Committee guidelines. Six-week-old female BALB/c mice (NCI, Raleigh, NC) were used in all studies. To prepare the oral inoculum, the proper dose of bacteria was suspended in PBS to 100 μl of inoculum per mouse. The entire bacterial suspension then was deposited into the stomach by oral gavage.

**Isolation of murine colonic lymphocytes.** Mice received oral bacterial treatment at 24-h intervals for 3 days. On the fourth day, 96 h after the primary dose, mice were euthanized via carbon dioxide inhalation and thoracotomy. Colon was harvested and placed in a petri dish containing PBS with 1 mM dithiothreitol (DTT). The colon was opened longitudinally and cut into 0.5-cm sections, which then were stirred in digestion medium at 37°C for 45 min. Digestion medium consisted of RPMI 1640 (Invitrogen), 1% (vol/vol) HEPES (Invitrogen), peni...
Flow-cytometric analysis of colonic lymphocytes. To evaluate percentages of B-cell populations, freshly isolated colonic lymphocytes underwent Fe receptor blocking (CD16/Cd32; clone 2,4G2; BD Biosciences) and then were stained with CD45-FITC (clone 30-F11; BioLegend), CD19-Pacific Blue (clone 30-F11; BioLegend), and CD38-PE-Cy7 (clone HIT2; BioLegend) for 20 min on ice. Cells were fixed for 5 min in 4% paraformaldehyde and analyzed on an LSR II flow cytometer with FACS Diva software (BD Biosciences).

To analyze the percentage of IL-17- and Foxp3-positive lymphocytes, cells were cultured with monensin (BioLegend) for 4 h, washed, and stained with CD45-FITC (clone 30-F11; BioLegend) and CD4-PE-Cy7 (clone RM4-5; BioLegend) in the presence of an Fe receptor blocking reagent (CD16/Cd32; BD Biosciences). Cells were fixed and permeabilized with the Foxp3 staining buffer set (eBioscience, San Diego, CA) and subsequently stained with IL-17-APC (clone TC11-18H10.1; BioLegend) and Foxp3-PE (clone 1502D; BioLegend). Analysis was performed on an LSR II flow cytometer with FACS Diva software (BD Biosciences).

Harvesting and preparation of fecal samples. Fecal samples were harvested and homogenized in a solution of PBS-10% goat serum for 30 min. Samples then were centrifuged, and 75 μl of supernatant was removed and stored at −80°C for downstream enzyme-linked immunosorbent assay (ELISA) testing. The remaining slurry was vortexed, and samples were taken for the quantification of the bacterial population. To quantify dosages, bacteria were grown in duplicate overnight in anaerobic conditions at 37°C on Rogosa agar containing erythromycin (5 μg/ml).

Colorimetric ELISA for murine antibody detection. L. gasseri strains ATCC 33323 and NCK1423 were grown in liquid MRS medium overnight at 37°C. Cultures were grown to an OD600 of 0.4, centrifuged, and resuspended in PBS. One hundred μl of bacterial suspension were mixed with 10 μl of each dilution (polyclonal; Bethyl, Montgomery, TX) was diluted 1:50,000 in dilution buffer as a secondary detection reagent for serum IgG, while goat anti-mouse IgA-HRP (polyclonal; Thermo Fisher Scientific) was diluted 1:10,000 in dilation buffer as a secondary detection reagent for serum IgG, while goat anti-mouse IgA-HRP (polyclonal; Bethyl, Montgomery, TX) was diluted 1:50,000 in dilation buffer as a secondary detection reagent for fecal IgA. One hundred μl of diluted secondary antibody was added to wells and allowed to incubate for 1 h at room temperature. Plates were washed five times in wash buffer and then developed with TMB substrate colorimetric ELISA for murine antibody detection. (A) TLR activation was determined by NF-κB induction using the embryonic alkaline phosphatase (SEAP)-expressing pNiFty reporter plasmid. SEAP activity in coculture supernatants was detected by a luminometer after development with the SEAP reporter gene assay per the manufacturer’s instructions. Data are presented as the average of duplicates from one experiment. Graphs are representive of three separately performed experiments. (B) Soluble bacterial lysates (10 μg) from L. gasseri ATCC 33323 (lane 2), NCK 1785 (lane 3), or FliCP (lane 4) were separated by electrophoresis on a polyacrylamide gel. Proteins were transferred to a PVDF membrane, incubated with anti-flagellin antibody followed by goat anti-mouse HRP, and then visualized by chemiluminescence. Molecular mass markers (lane 1) are 60, 50, and 40 kDa (Magic Mark XP; Invitrogen). Lanes 6, 7, and 8 show recombinant flagelin FlIC (InvivoGen) at 25, 50, and 100 ng, respectively.

RESULTS

Lactobacillus primarily activates TLR2/6 with secondary activation through TLR2. Although many studies have shown that lactobacilli activate TLR2, the complete TLR activation profiles of Lactobacillus species are unknown. We therefore determined the TLR activation profile of L. gasseri ATCC 33323 and NCK1785 (Table 1). TLR-mediated NF-κB production was determined using a SEAP-secreting pNiFty reporter plasmid transfected into HEK293 cells expressing individual human TLR1 to TLR9 or heterodimers of TLR1/2 and TLR2/6. L. gasseri strongly activates TLR2/6 with lesser activation through TLR2 alone (Fig. 1A). No significant activation of TLR3, TLR4, TLR5, TLR7, TLR8, or TLR9 was noted.
Similar results were observed when HEK cells were treated with *L. acidophilus* NCFM (data not shown), suggesting that the TLR activation profile of *L. gasseri* was not specific to that species but, rather, was indicative of a general *Lactobacillus* profile.

To determine whether recombinant bacteria transformed with pTRK563 activate TLR9 due to additional bacterial CpG motifs, ATCC 33323, NCK 1785, and FliCP were evaluated as whole cells and cell lysates, with and without liposomal encapsulation. Regardless of the preparation, no activation of human or murine TLR9 through the type B pathway could be demonstrated (data not shown). Additionally, the analysis of CpG motifs in *L. gasseri* ATCC 33323 with Clone Manager software (Sci-Ed, Cary, NC) showed a low frequency of genomic CpG dinucleotides (2.34%), which has been associated with low levels of TLR9 activation (5). From these results we concluded that neither the *L. gasseri* genome nor the plasmid backbones used in these studies intrinsically expanded the TLR activation repertoire of *L. gasseri* to include TLR9.

**FIG. 2.** Evaluation of TLR activation of live versus killed *L. gasseri*. HEK293 cells stably transfected with human TLRs as indicated were cocultured with live or killed lactobacilli (*L. gasseri* ATCC 33323 or FliCP) at different bacterial concentrations. The bacterial concentration (x axis) is denoted as a ratio of HEK293 cells to bacteria. Activation values are relative to those of the positive-control synthetic ligand (recFLA-ST; Invivogen). Data are presented as the averages from three independent experiments. Error bars denote means ± standard errors of the means. An asterisk designates significance compared to all other treatments (*P* < 0.05).

Addition of *fliC* to *L. gasseri* results in activation of TLR5 in addition to TLR2/6 and TLR2. It has been clearly shown that the simultaneous activation of multiple Toll-like receptors can lead to amplified downstream innate and adaptive immune responses (17). TLR5 is expressed on the basolateral surface of intestinal epithelial cells (9), CD11c⁺ lamina propria DCs (31), and the follicle-associated epithelium of Peyer’s patches in mice (2), making it a ubiquitous target for orally delivered adjuvants. To expand the TLR activation profile of *L. gasseri* to include TLR5, we transformed *L. gasseri* with an expression plasmid, pFliCP, encoding the sequence of flagellin (*fliC*) from *S. Typhimurium* (Fig. 1B). Figure 1C shows that cell lysates from *fliC*-transformed *L. gasseri* activate TLR5 in addition to TLR2/6.

**Live L. gasseri induces greater Toll-like receptor activation than dead lactobacilli.** Others have demonstrated differences in the immunogenicity of live versus killed recombinant bacteria (4, 6). We tested whether live or UV-killed lactobacilli were more efficient at activating Toll-like receptors and whether the dosage of killed bacteria influenced the degree of TLR activation. A multiplicity of infection (MOI) of 50 was used. Based on preliminary titration studies, this was the maximum dose of live bacteria that was not cytotoxic to HEK293 cells (data not shown). This dose of live bacteria was compared to four doses of killed bacteria (1:25, 1:50, 1:100, and 1:200) on cell lines expressing TLR2/6 or TLR5 (Fig. 2). At the 1:50 dose, live bacteria always elicited a greater NF-κB response than killed bacteria. At no dose did killed bacteria statistically surpass the ability of live bacteria to increase NF-κB production. In our system, live bacteria seem to have a greater intrinsic capacity to activate TLRs than dead bacteria.

**Recombinant L. gasseri induces phenotypic maturation of human myeloid DCs.** To establish how genetically modified lactobacilli influence dendritic cell maturation and coreceptor expression, the mDC surface expression of major histocompat-
ibility complex class II (MHC-II), CD80, and CD86 was evaluated by flow-cytometric analysis. Freshly isolated human mDCs pulsed with ATCC 33323, NCK 1785, or FliCP trended toward an increased population of MHC-II$^+$ CD80$^+$ CD86$^+$ cells (11.0, 11.6, and 11.6%, respectively) compared to that of untreated control mDCs (3.4%) (Fig. 3). The percentage of MHCII$^+$ CD80$^+$ CD86$^+$ cells was higher in the two mDC groups pulsed with genetically modified L. gasseri (8.2 and 9.0%) compared to that of the untreated control mDC population (2.3%) ($P < 0.005$). The proportion of MHCII$^+$ CD80$^+$ CD86$^+$ cells in mDCs pulsed with genetically modified L. gasseri was significantly lower than that of MRS-treated control mDC populations.

**Recombinant L. gasseri induces proinflammatory cytokine/chemokine release and a high IL-10/IL-12 ratio in myeloid DCs.** We next sought to determine how wild-type and recombinant L. gasseri strains influence cytokine and chemokine production by human mDCs. Lactobacilli were incubated with mDCs for 24 h, and then culture supernatants were quantitatively assayed for cytokine and chemokine levels. Generally, incubation with any L. gasseri treatment resulted in a statistically significant increase in proinflammatory cytokine production by dendritic cells, including IL-1β, IL-1Ra, IL-6, IFN-γ, TNF-α, GM-CSF, and G-CSF (Fig. 4A and Table 2). The addition of lactobacilli to DCs also resulted in a statistically significant increase in chemokine (IL-8, MIP1-α, and MIP1-β) secretion (Fig. 4B and Table 2). Finally, all L. gasseri treatments resulted in the increased production of IL-10 compared to that of controls ($P < 0.0001$), whereas IL-12 production was not significantly influenced by bacterial treatment (Fig. 4C). These results suggest that L. gasseri has a role both in promoting a mixed cytokine response by DCs and in the recruitment of immune cells.

**L. gasseri treatment leads to a diversification of the colonic B-cell population in mice.** Recent studies have shown that individual probiotic bacteria can influence the mucosal immune response, specifically at immune induction sites in the murine small intestine (3). We sought to determine the role of L. gasseri, generally, and genetically modified lactobacilli, specifically, in the colonic lamina propria, an effector site of the mucosal immune system. We further wanted to determine how L. gasseri expressing the FliC adjuvant would influence plasma cell populations, a change which could affect downstream antibody production after vaccine delivery. Mice were orally administered $1 \times 10^9$ CFU of bacteria for three consecutive days prior to euthanasia. Upon euthanasia, mononuclear cells were isolated from colonic tissue and stained for CD45 (to identify all leukocytes), CD19, and CD38. Mature plasma cells (CD19$^-$ CD38$^+$) predominated in PBS-treated mice, while all three L. gasseri-treated groups showed the diversification of the colonic B-cell population compared to that of PBS controls (Fig. 5). This was characterized by a significantly increased percentage of B cells (CD19$^+$ CD38$^+$) and B cells in transition to plasma.
cells (CD19<sup>+</sup> CD38<sup>+</sup>) and was most pronounced in mice treated with wild-type L. gasseri.

**Wild-type and genetically modified L. gasseri affect the T<sub>reg</sub>/Th17 ratio in the murine colon.** We next sought to determine the effect of L. gasseri treatment on Foxp3<sup>+</sup> and IL-17<sup>+</sup> cell populations in the murine colon. Lymphocytes were isolated from the colon after three consecutive days of bacterial treatment. Staining for CD4<sup>+</sup> Foxp3<sup>+</sup> cells revealed that treatment with L. gasseri significantly increased the percentage of regulatory T cells (T<sub>reg</sub>) in the murine colon compared to that of PBS treatment alone (Fig. 6B). In contrast, treatment with genetically modified lactobacilli significantly decreased the T<sub>reg</sub> population of the colon compared to those of both WT- and PBS-treated mice.

IL-17<sup>+</sup> cells were identified by intracellular cytokine staining. Compared to PBS treatment, we observed a statistically significant decrease in IL-17<sup>+</sup> cells in mice treated with L. gasseri containing an empty expression plasmid (Fig. 6C). Treatment with wild-type L. gasseri and FliC also led to a decrease in the percentage of IL-17<sup>+</sup> cells, but this decrease was not significant.

**Wild-type and genetically modified L. gasseri survive but do not colonize the intestinal tract.** The determination of the optimal dose of a live, orally delivered, bacterium-based vaccine must consider whether the bacteria survive the acid environment of the stomach and bile in the proximal small intestine, whether the bacteria colonize the gastrointestinal tract, and whether the host will mount an anti-vector response upon multiple exposures. To empirically assess the effect of dose on persistence, BALB/c mice were orally gavaged with L. gasseri at five doses: 1 × 10<sup>7</sup>, 1 × 10<sup>8</sup>, 1 × 10<sup>9</sup>, 1 × 10<sup>10</sup>, and 1 × 10<sup>11</sup> CFU. At 24 h, the quantity of bacteria found in the feces was directly related to the inoculation dose (Fig. 7A). Fecal shedding continued to 48 h only in those animals that received ≥10<sup>7</sup> CFU. L. gasseri was never recovered from the cecum, small intestine, or large intestine upon the culture of intestinal tissue and luminal contents at necropsy (72 h postinoculation; data not shown). We conclude that 1 × 10<sup>10</sup> CFU of L. gasseri is the optimal dose for mice, because it persists for up to 48 h and can be readily concentrated into a 100-μl dose for oral gavage.

A common concern with vectored vaccines is the induction of an anti-vector immune response. In the case of lactobacilli, this may not only reduce the efficacy of subsequent boosts by affecting vector persistence but also could result in an anti-commensal response, such as that associated with inflammatory bowel disease. To assess the likelihood of an anti-L. gasseri response, we inoculated mice with 1 × 10<sup>10</sup> CFU of L. gasseri at 2-week intervals for a total of six immunizations. Although variation existed between time points within each inoculation group, no net increase or decrease in the fecal shedding of control or recombinant L. gasseri was observed over time (Fig. 7B). Thus, a multiboost inoculation approach does not alter host-bacterium interactions in a way that affects the magnitude of fecal shedding or the persistence (beyond 24 h) of the L. gasseri vector in the host. Furthermore, we did not detect mucosal or systemic antibodies against bacterial treatments (Fig. 7C), suggesting that immunization with an L. gasseri vector will not prompt an anti-vector immune response.

**DISCUSSION**

The goal of this study was to assess key immunomodulatory characteristics of wild-type and recombinant L. gasseri, with the specific aim of developing Lactobacillus gasseri as an orally delivered vaccine vector. We determined that the TLR activation profile of L. gasseri is limited to TLR2 and the TLR2/6 heterodimer but could be expanded to include TLR5 by the expression of FliC. We fully characterized the activation of human myeloid dendritic cells by recombinant and wild-type L. gasseri and comprehensively assessed cytokine and chemokine expression. Lastly, we investigated the immunomodulatory effects of orally administered L. gasseri on colonic B- and T-cell populations. Taken together, these results set the stage for

**FIG. 4.** Cytokine expression by L. gasseri-pulsed mDCs. (A) Cytokine production by mDCs. (B) Chemokine production by mDCs. (C) IL-10/IL-12 ratio. Data that are significantly different (P < 0.05) from those of the MRS control are represented by an asterisk, significant difference from MRS and FSL-1 by two asterisks, and significant difference from MRS, FSL-1, and flagellin by three asterisks. Only data within the upper and lower limits of the standard curve were included in the statistics. The second quartile of the box-and-whisker plot represents median values. n = 3 to 6 donors per group.

| Analyte and significance difference level | P value |
|------------------------------------------|---------|
| Significant                               |         |
| IL-2                                     | 0.0378  |
| IL-4                                     | 0.0021  |
| IL-6                                     | 0.0014  |
| IL-8                                     | 0.0418  |
| IL-10                                    | 0.0002  |
| GM-CSF                                    | 0.0053  |
| IFN-γ                                    | <0.0001 |
| TNF-α                                    | 0.0064  |
| IL-1Rα                                    | 0.0005  |
| IL-1β                                    | 0.0091  |
| G-CSF                                    | 0.0002  |
| MCP1                                     | <0.0001 |
| MIP1-β                                   | 0.0347  |
| MIP1-α                                   | 0.0125  |
| Nonsignificant                           |         |
| IL-5                                     | Below detection |
| IL-7                                     | 0.4466  |
| IL-12                                    | 0.3685  |
| IL-13                                    | 0.6616  |
| IL-17                                    | 0.3401  |
| IL-9                                     | 0.2491  |
| IL-15                                    | 0.5191  |
| Eotaxin                                  | 0.5056  |
| FGF basic                                | 0.7377  |
| IP-10                                    | 0.4351  |
| PDGF                                     | 0.4571  |
| RANTES                                   | 0.1024  |
| VEGF                                     | 0.4338  |
further in vivo immunogenicity studies with *L. gasseri* expressing flagellin in combination with vaccine antigens of interest.

One of the main advantages of biologic vaccine vectors is their inherent immunostimulatory properties. *Lactobacillus* sp. have been shown to interact with dendritic cells through NOD2, DC-SIGN, and TLR2 (18, 25). Here, we have further characterized the TLR binding activity of *L. gasseri* to demonstrate the preferential activation of the TLR2/6 heterodimer with the lesser activation of TLR2 alone. Given that lipoproteins from Gram-positive bacteria have only two lipid chains, which together form the diacylated motif known to be essential for the dimerization of TLR2/6 (13, 23, 29), it is not surprising that *L. gasseri* preferentially activates this Toll-like receptor heterodimer.

Few studies have reported the effects of the specific activation of TLR2/6 as an adjuvant. In a mouse model of HSV infection, the vaginal pretreatment of mice with the TLR2/6 ligand FSL-1 produced a statistically significant increase in IL-12 and IFN-γ production (26). FSL-1 treatment of macrophages was shown to increase the phagocytosis of bacteria and upregulate DC-SIGN, while the FSL-1 treatment of dendritic cells induced maturation (14, 16). The net effect of *L. gasseri* TLR activation will depend on the cell types in the mucosa expressing TLR2 and the heterodimeric partner, TLR6, and the capacity of *L. gasseri* to access those cell types.

A considerable body of evidence has shown flagellin to be a potent adjuvant for vaccination (20), and the immunogenic potential of *Lactobacillus casei* expressing flagellin has been previously reported (11, 12). In the mucosa, TLR5 signaling promotes the rapid production of IL-17 and IL-22, leading to

FIG. 5. Colonic lamina propria B-cell populations in lactobacillus-treated mice. Mice were orally gavaged as indicated (x axis) for three consecutive days prior to euthanasia. (A) Gating strategy for B-cell staining. (B) Percentages of colonic B-cell populations. Cells were stained with CD45-FITC, CD19-PacBlue, and CD38-PE-Cy7. #, significance compared to data for WT-treated mice; *, significance compared to data for PBS-treated mice; **, significance compared to data for vector-treated mice. $P < 0.05$. $n = 3$ to 6 animals per group.
downstream Th17 cell development (33). Our data show that oral treatment with FliCP did not increase the Th17 cell population of the murine colon. In fact, despite statistical differences in Th17 percentages among treatment groups, percentages were quite low and most likely were not biologically significant. TLR5 is expressed on the extracellular surface of immune cells and the basolateral surface of intestinal epithelial cells, thus the intracellular flagellin expressed by FliCP may not be available to bind TLR5 under these *in vivo* conditions. This biological sequestration of flagellin may account for the low Th17 percentage we observed compared to those of other studies involving TLR5 signaling (33). The cell surface expression of FliC could increase TLR5 binding, but it is uncertain whether the surface-expressed FliC could survive gastric acid and bile salts in transit to immune induction sites of the intestine. Further studies involving cell surface-associated flagellin would be necessary to resolve these questions.

The cytokine-inducing effects of many strains of lactobacilli on DCs have been reported but typically focus on Th1/2-polarizing cytokines such as IL-10 and IL-12. Given the complexity of the mucosal immune response, we chose to take a more comprehensive look at cytokine and chemokine expression by human myeloid dendritic cells. The synthetic ligand for TLR2/6 (FSL-1) was used as a positive control. *L. gasseri* induced a broader spectrum and greater magnitude of cytokine/chemokine expression than FSL-1 alone, suggesting that the immunostimulatory effects of *L. gasseri* involve more than just TLR2/6 activation. These results are similar to those reported by Zeuthen et al. in studies employing *L. acidophilus* (35).

The cytokine/chemokine profile induced by *L. gasseri* generally can be interpreted as proinflammatory, with the notable exceptions of low IL-12 expression concomitant with increased IL-10 expression. Both the upregulation and downregulation of IL-12 expression by lactobacilli have been reported to be mediated through TLR2 or NOD2 (30, 35). The conflicting results are due to the *Lactobacillus* species used, the cell type evaluated, and the temporal order of TLR2 and NOD2 stimulation. Complicating this further is the complexity of NOD2, which is reported as both a proinflammatory and anti-inflammatory pattern recognition receptor (35). However, Mohamadezadeh et al. clearly showed that although the oral administration of adjuvanted *L. gasseri* resulted in a high IL-10/IL-12 ratio, the net effect of the bacterial treatment was significant protection against *B. anthracis* infection (21). Although the relative amount of IL-10 and IL-12 often is cited as a predictor of inflammatory status, the results from the Mohamadezadeh et al. study suggest that the IL-10/IL-12 ratio cannot be relied upon alone to predict vaccine effectiveness. Thus, the mixed inflammatory profile resulting from *L. gasseri* treatment should not deter the usage of *L. gasseri* as a vector. The cumulative effect of the cytokine milieu will need to be studied in more detail to determine its consequences on downstream immune responses.

We found that *L. gasseri* survived passage through the hos-
The intestinal environment of the GI tract and could be cultured from feces at 24 to 48 h postinoculation. This might be important, since TLR activation by live bacteria was, according to our results, superior to that of dead bacteria. Supporting this, a study by Grangette et al. showed that lactic acid bacterial strains that did not persist in the GI tract were less efficient at initiating a protective humoral immune response in the host than bacteria that do persist in the GI tract (10). However, the long-term colonization of recombinant L. gasseri in the gastrointestinal tract may not be optimal for several reasons. First, there may be unintended consequences if recombinant L. gasseri were to compete with and potentially alter the host microbiome. Additionally, the long-term shedding of a genetically modified organism is unlikely to achieve regulatory approval. Lastly, colonization may not be compatible with immunogenicity. A recent study by Round et al. (27) clearly demonstrated that commensal colonization is dependent on T<sub>reg</sub> induction by the bacteria, whereas in our case, T<sub>reg</sub> induction likely would
undermine attempts to tip the balance from tolerance to immunity induction by a commensal vaccine vector. Interestingly, we found that recombinant L. gasseri significantly reduced the percentage of $T_{reg}$ in the colonic lamina propria. This did not lead to clinical or gross pathological abnormalities or to the induction of an anti-Lactobacillus antibody response.

To our knowledge, no previous studies have reported the effect of oral Lactobacillus treatment on B-cell populations of the murine colon. Our studies showed that treatment with L. gasseri significantly increased the percentage of cells that had not yet differentiated to mature plasma cells. This may explain the association of increased mucosal IgA with oral L. gasseri treatment (24). Whether L. gasseri in general and FliCP in particular affect the magnitude or kinetics of plasma cell induction of an anti-Lactobacillus antibody response. Interestingly, the mechanisms of immune modulation by a commensal such as L. gasseri is essential for its rational development as an oral vaccine vector.

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