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SHORT COMMUNICATION

Gut commensal *Lactobacillus reuteri* 100-23 stimulates an immunoregulatory response

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***Lactobacillus reuteri* 100-23 is a bacterial commensal of the gastrointestinal tract of mice. Previous studies have shown that colonization of the murine gut by this strain stimulates small-bowel enterocytes to produce proinflammatory cytokines. This is associated with a mild, transitory inflammatory response 6 days after inoculation of formerly *Lactobacillus*-free animals. The inflammation subsides by 21 days after colonization, although lactobacilli continue to be present in the bowel. To determine the immunological mechanisms that underpin tolerance to bowel commensals, we investigated cytokine responses of dendritic cells and T cells after exposure to cells of *L. reuteri* 100-23. Interleukin-10 (IL-10), IL-2 and transforming growth factor-β (TGF-β) concentrations in supernatants of cultured immune cells, as well as the results of proliferative assays of mesenteric lymph node (MLN) cells and quantification of Foxp3-positive cells in MLN and spleen, indicated that *L. reuteri* 100-23 stimulated the development of an increased number of regulatory T cells.**
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The digestive tracts of mammals contain biodiverse bacterial communities, the members of which are referred to as commensals.[1] The forestomach of the gastric region of mice is lined with a keratinized, stratified squamous epithelium, and commensal lactobacilli, such as *Lactobacillus reuteri* strain 100-23, attach directly to this epithelium. They proliferate to form a layer (biofilm) of cells, and as they are carried on squames into the digesta, they can be detected in substantial numbers throughout the remainder of the digestive tract.[2] A unique mouse colony has been developed, which, unlike conventional mice, does not harbor lactobacilli as commensals of the digestive tract.[3] Although *Lactobacillus* free, the mice harbor a biodiverse bacterial community in the large bowel. The mucosa is therefore conditioned by exposure to bacteria, characteristic of the murine bowel biome, but is naive with respect to the influences of lactobacilli. These mice provide a defined system by which the impact of lactobacilli on the host immune response can be studied.

Oral administration of *L. reuteri* 100-23 cells to adult *Lactobacillus*-free mice results in colonization of the forestomach by lactobacilli and a mild inflammatory response in the ileal mucosa 6 days after inoculation.[4] Transcription of genes encoding interleukin (IL)-1α and IL-6 is increased in small-bowel enterocytes at this time. This coincides with the development of a maximal population of lactobacilli in the ileum. Although the numbers of lactobacilli remain at this same constant level throughout the remainder of the animal's life, inflammation is transient and resolves by 21 days after inoculation, at which time the IL gene expression of enterocytes returns to baseline.[4] It seems, therefore, that the innate immune response to the presence of *L. reuteri* 100-23 is downregulated with time, but by an as yet unknown mechanism. Antibodies that react with a large protein on the surface of *L. reuteri* 100-23 cells, indicative of an adaptive immune response, are present in sera of mice that have been colonized by the bacteria, but not in the sera of noncolonized mice (GW Tannock, unpublished). Overall, however, bowel commensals do not invoke an immune response of pathological consequences under normal circumstances. The mechanisms by which this is achieved are largely unknown. Thus, we used the *L. reuteri* 100-23/*Lactobacillus*-free mouse paradigm to test the impact of this commensal on immunoregulation. We reasoned that, as front-line antigen-presenting cells of bowel mucosa, the way in which dendritic cells (DCs) react to *Lactobacillus* cells would be pivotal in directing the nature of the adaptive immune response.

RESULTS AND DISCUSSION

Dendritic cells not only initiate responses in naive T cells but also influence the nature of the subsequent immune response, regulatory or stimulatory. Therefore, we first determined the cytokine response of DCs after exposure to heat-killed *Lactobacillus* cells. Cytokine concentrations were measured in supernatants from bone marrow-derived DC (BMDC) cultures exposed to heat-killed cells of *L. reuteri* 100-23. The supernatants were collected 12, 24 and 48 h after exposure. BMDCs were used in the experiments because they were more readily accessible for harvest than the relatively low numbers of these cells associated with bowel mucosa. Although BMDCs may not respond to antigens in exactly the same way as mucosal DCs, they can

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still serve as indicators of cytokine responses when screening for the effects of bacterial strains.[5] Exposing BMDCs to heat-killed *L. reuteri* 100-23 strongly favored the production of IL-10 rather than IL-12 when compared with heat-killed *Lactobacillus johnsonii* 100-5, *L. johnsonii* 100-33 and *L. johnsonii* 21 (Figures 1a and b). This suggested that *L. reuteri* 10-23 induced a regulatory rather than a stimulatory DC phenotype.[6]

We next determined the downstream effect of BMDC exposure to strain 100-23 on an antigen-specific T-cell response. BMDCs pulsed with ovalbumin and then exposed to heat-killed *L. reuteri* 100-23 cells were cocultured with splenic T cells from ovalbumin T-cell receptor, transgenic DO11.10 mice. The IL-2 production measured in culture supernatants was less than that from BMDC cultures that were ovalbumin pulsed but not exposed to heat-killed *L. reuteri* 100-23 cells (Figure 1c). Transforming growth factor-β production, in contrast, was greater in preparations treated with strain 100-23 (Figure 1d) relative to controls. The generation of Treg cells requires transforming growth factor-β[7] and these cells have been shown to

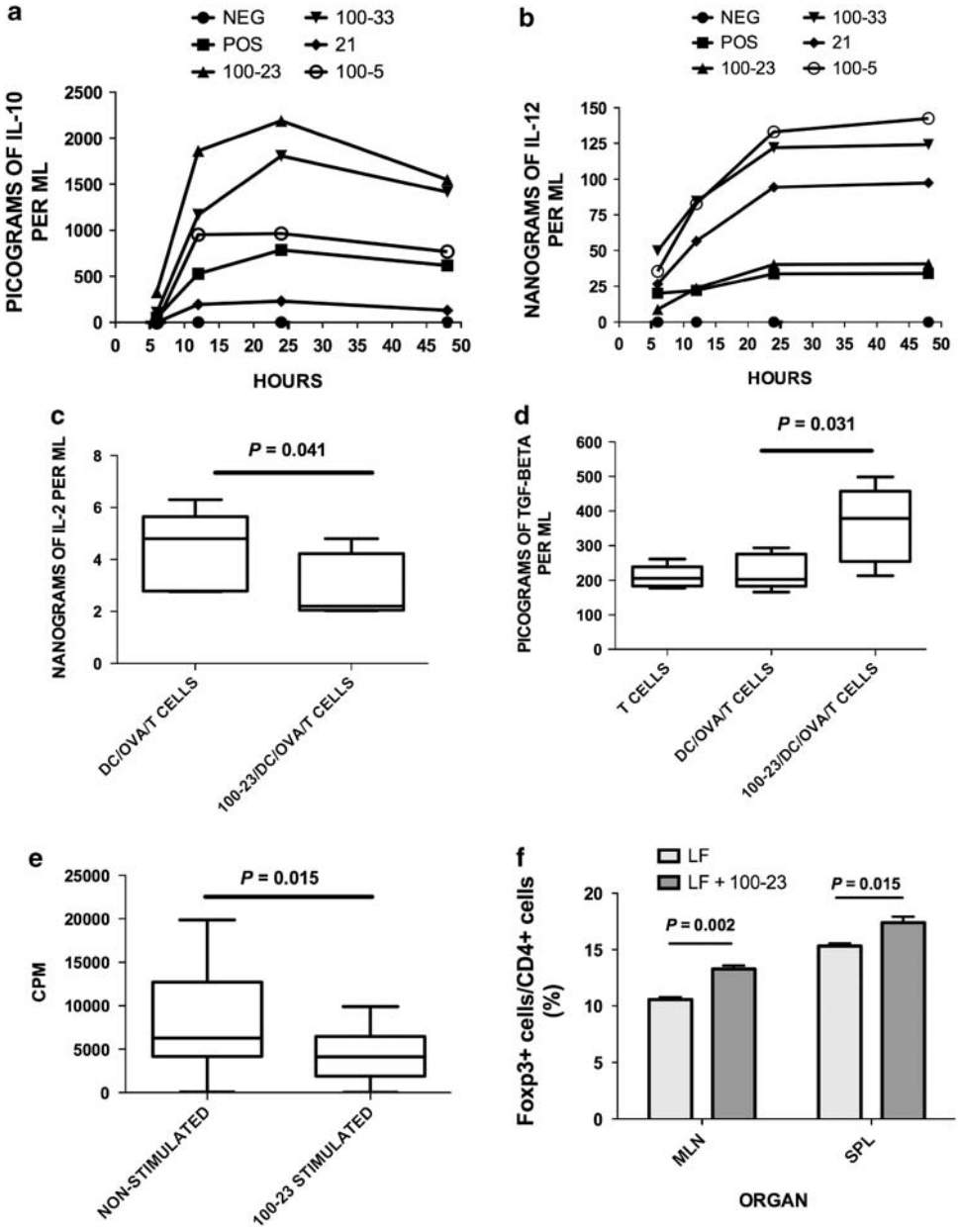

Figure 1 (**a** and **b**) Temporal cytokine production by bone marrow-derived dendritic cells (BMDCs) exposed to heat-killed cells of *L. reuteri* 100-23 or *L. johnsonii* strains 100-5, 100-33 or 21. POS, BMDCs exposed to lipopolysaccharide; NEG, negative control. (**c** and **d**) Cytokine production by cocultures of BMDCs and DO11.10 T cells. DC/ovalbumin (OVA)/T cells, BMDCs not exposed to lactobacilli; 100-23/DC/OVA/T cells, BMDCs exposed to heat-killed cells of *L. reuteri* 100-23; T cells, DO11.10 cells only. Medians (horizontal line), 25–75% confidence limits (box) and ranges (vertical bars) are shown. *N*=6 values. (**e**) Proliferation of mesenteric lymph node (MLN) cells cocultured with splenic DCs. Non-stimulated, splenic DCs not exposed to lactobacilli; 100-23 Stimulated, splenic DCs exposed to heat-killed cells of *L. reuteri* 100-23. *N*=20 values per group. Medians (horizontal line), 25–75% confidence limits (box) and ranges (vertical bars) are shown. (**f**) Proportions of CD4-positive T cells that were Foxp3 positive. MLN, cells harvested from mesenteric lymph nodes; SPL, cells harvested from spleens; LF, *Lactobacillus*-free mice; LF+100-23, ex-*Lactobacillus*-free mice colonized by *L. reuteri* 100-23. Means and s.e. of the mean are shown. *N*=6 values per group.

inhibit the production of IL-2.[8] They may also deplete IL-2 concentrations by competing with non-Treg for this cytokine.[9] Our data suggested, therefore, that DCs exposed to heat-killed *L. reuteri* 100-23 preferentially induced the development of Treg.

To investigate this further, we utilized *Lactobacillus*-free mice. Mesenteric lymph node (MLN) cells were harvested from 12-week-old *Lactobacillus*-free mice and cocultured with splenic DCs, also isolated from these mice, which had been exposed to heat-killed *L. reuteri* 100-23 cells. Control cultures contained nonstimulated DCs. MLN cells cultured with DCs stimulated with heat-killed *L. reuteri* 100-23 proliferated to a lesser extent than did those containing nonstimulated DCs (Figure 1e). This was consistent with the induction of Treg cells within the population.[8]

Finally, we compared the percentages of Foxp3+ cells in spleen and MLN cells harvested from mice colonized by *L. reuteri* 100-23 with those of *Lactobacillus*-free mice. The percentage of Foxp3+ T cells from mice colonized by *L. reuteri* 100-23 was found to be significantly greater than that from *Lactobacillus*-free mice in both MLN and spleen cell populations (Figure 1f). Therefore colonization of the gastrointestinal tract of mice by this *Lactobacillus* strain increased the size of the Treg population. This finding not only supports general observations by others that commensal bacteria have a role in the generation of Treg[10–13] but, by using a unique model, also identifies a specific bacterial strain involved in the induction of these cells.

All of our observations were consistent with the view that *L. reuteri* 100-23 stimulates an immunoregulatory response in the murine host, which suppresses immune responses directed against it in the bowel, thus enabling the bacteria to persist in the gut and maintain a commensalistic relationship with the murine host. Future studies of this intriguing relationship might include the discovery of molecules, presumably associated with the surface of *Lactobacillus* cells, that mediate the pertinent immunological phenomena.

METHODS

Bacterial preparations used in *ex vivo* experiments

Lactobacillus reuteri 100-23, *L. johnsonii* 100-5, *L. johnsonii* 100-33 and *L. johnsonii* 21 were originally isolated from the gut of conventional mice.[14] Preparations containing lactobacilli for use in *ex vivo* experiments were prepared from overnight, anaerobic cultures of bacteria (37 °C in Lactobacilli MRS medium (Becton Dickinson, Franklin Lakes, NJ, USA) in which glucose was replaced with sucrose). *Lactobacillus* cells harvested by centrifugation from the broth cultures were washed four times in sterile phosphate-buffered saline (pH 7.4) and finally suspended in sterile deionized water. The bacterial cell suspensions were exposed to 80 °C for 2 h, and loss of cell viability was checked by culture on MRS agar plates. The cell suspensions were lyophilized for 48 h and stored at 4 °C.

Mice

Specific pathogen-free, BALB/c and DO11.10 female mice,[15] aged 6–16 weeks, were obtained from the Hercus Taieri Research Facility (HTRF), University of Otago, New Zealand, and were housed under specific pathogen-free conditions. Male and female BALB/c, *Lactobacillus*-free mice (6-weeks old) were obtained from the colony maintained at the Department of Microbiology and Immunology, University of Otago.[3] The *Lactobacillus*-free status of mice was confirmed by the absence of growth on Rogosa SL agar medium incubated anaerobically for 48 h at 37 °C before experimentation. All experimental protocols were approved by the University of Otago Animal Ethics Committee AEC ET19/05 and AEC 60/03.

Dendritic cells

Dendritic cells were generated from bone marrow as described previously.[16] Day 6 nonadherent cells (1×10^6 cells ml^{-1}) were cultured with 10 μg ml^{-1} bacterial cells or 10 μg ml^{-1} bacterial lipopolysaccharide (*Escherichia coli* O26:B6; Sigma Chemical Company, St Louis, MO, USA), or with uninoculated medium. Culture supernatants were collected at 12, 24 and 48 h after stimulation for cytokine analyses.

Cocultures of DCs and T cells

Splenic DO11.10 CD4 and CD8 T cells were sorted to >98% purity after labeling with antigen-presenting cell- or fluorescein isothiocyanate-conjugated rat anti-mouse mAb (clones RM4-5 and 53-6.7, respectively; BD Pharmingen, San Diego, CA, USA), using a FACSAria flow cytometer (Becton Dickinson). These T cells (2×10^6 cells ml^{-1}) were cocultured with BALB/c BMDC stimulated with 100 μg ml^{-1} ovalbumin (chicken egg albumin; Sigma Chemical Company; 24 h) and 10 μg ml^{-1} bacterial cells or lipopolysaccharide (8 h) in complete Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 20 ng ml^{-1} recombinant murine granulocyte macrophage-colony-stimulating factor for 72 h. Supernatants were collected for cytokine analyses.

Cytokine assay

Interleukin-10, IL-12p70, transforming growth factor-β, IL-2 and interferon-γ concentrations were quantified by enzyme-linked immunosorbent assay according to the BD PharMingen cytokine ELISA protocol.[17] Capture and detection antibodies for these sandwich assays were obtained from BD PharMingen, and recombinant cytokines to generate standard curves were purchased from R&D Systems (Minneapolis, MN, USA).

MLN cell proliferation assays

Spleen and MLN cells were harvested from mice. CD11c+ cells (DC) were selected from spleen cells using MACS mouse CD11c (N418) microbeads (Miltenyi Biotec, Auburn, CA, USA) and an AutoMACS separator, according to the manufacturer's instructions. DCs were pulsed with 10 μg ml^{-1} bacterial cells or medium overnight, then cocultured with MLN cells from *Lactobacillus*-free mice at 1:10 ratio for 72 h, no less than three times. Cell proliferation was quantified by tritiated thymidine incorporation as described previously.[17]

Lactobacillus-free mouse experiments

Lactobacillus-free mice were maintained in isolators throughout experiments, as described previously.[2] Six *Lactobacillus*-free BALB/c mice were inoculated ($\sim10^6$ *Lactobacillus* cells) by gavage on a single occasion at 6 weeks of age with *L. reuteri* 100-23 that had been cultured anaerobically in MRS medium overnight. Inoculated mice and matched noncolonized animals were killed 3 weeks later. Colonization by *L. reuteri* 100-23 was confirmed by culturing samples from the small and large intestine during necropsy. MLN and spleens were removed during necropsy and used for Foxp3 measurements. Experimental protocols were approved by the University of Otago Animal Ethics Committee (AEC 36/06).

Proportions of Foxp3-positive cells in MLN and spleens

Single-cell suspensions from spleens and MLN were labeled with antibodies to CD4, CD25 and Foxp3 following the instructions in a kit supplied by eBioScience (San Diego, CA, USA). The cells were analyzed using a flow cytometer (FACSCalibur, BD Biosciences) and BD CELLQuest software (San Jose, CA, USA).

Statistical analysis

Data were analyzed using the Mann–Whitney nonparametric test.

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