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Lactobacillus reuteri–induced Regulatory T cells Protect against an Allergic Airway Response in Mice

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***Rationale*: We have previously demonstrated that oral treatment with live *Lactobacillus reuteri* can attenuate major characteristics of the asthmatic response in a mouse model of allergic airway inflammation. However, the mechanisms underlying these effects remain to be determined.**

***Objectives*: We tested the hypothesis that regulatory T cells play a major role in mediating *L. reuteri*–induced attenuation of the allergic airway response.**

***Methods*: BALB/c mice were treated daily with *L. reuteri* by gavage. Flourescent-activated cell sorter analysis was used to determine $CD4^+CD25^+Foxp3^+$T cell populations in spleens following treatment with *L. reuteri* or vehicle control. Cell proliferation assays were performed on immunomagnetic bead separated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ T cells isolated from, ovalbumin naive, *L. reuteri* treated mice were transferred into ovalbumin-sensitized mice. Following antigen challenge the airway responsiveness, inflammatory cell influx and cytokine levels in bronchoalveolar lavage fluid of recipient mice were assessed.**

***Measurements and Main Results*: Following 9 days of oral *L. reuteri* treatment, the percentage and total number of $CD4^+CD25^+Foxp3^+$T cells in spleens significantly increased. $CD4^+CD25^+$ cells isolated from *L. reuteri*–fed animals also had greater capacity to suppress T-effector cell proliferation. Adoptive transfer of $CD4^+CD25^+$ T cells from *L. reuteri*–treated mice to ovalbumin-sensitized animals attenuated airway hyper-responsiveness and inflammation in response to subsequent antigen challenge.**

***Conclusions*: These results strongly support a role for nonantigen-specific $CD4^+CD25^+Foxp3^+$ regulatory T cells in attenuating the allergic airway response following oral treatment with *L. reuteri*. This potent immuno-regulatory action may have therapeutic potential in controlling the Th2 bias observed in atopic individuals.**

Keywords: commensal bacteria; Foxp3; airway inflammation; allergy

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Although there is evidence that exposure to certain commensal bacteria can reduce allergic responses the exact mechanisms behind these effects are still obscure.

What This Study Adds to the Field

This study provides evidence that oral treatment with a *Lactobacillus* strain induces regulatory T cells that can attenuate the allergic airway response in a nonantigen-specific manner.

Although significant attention has been focused on the role of the gut microbiota in gastrointestinal (GI) development, immune adaptation, and attenuation of GI inflammatory diseases, there is steadily increasing evidence that certain commensal bacteria may also be able to regulate immune responses outside the GI tract and, in particular, there is growing interest in the therapeutic potential of these organisms in allergic disorders.

Clinical trials have indicated that *Lactobacillus rhamnosus* GG may be effective in treatment and prevention of early atopic disease (1, 2) and *Lactobacillus fermentum* was shown to be beneficial in improving the extent and severity of atopic dermatitis in young children (3). Although long-term follow-up studies of children at high risk for developing allergy have not shown significant beneficial effects of commensals on the incidence of asthma (1, 4), there is evidence indicating that oral administration of certain organisms can modulate immune responses in the airway (5–7). In previous studies we demonstrated that oral treatment of adult, ovalbumin (OVA)-sensitized, mice with live *Lactobacillus reuteri*, but not *Lactobacillus salivarius*, significantly attenuated airway hyperresponsiveness (AHR) and inflammation following an inhaled antigen challenge (7). Furthermore, it has recently been shown that perinatal treatment with *Lactobacillus rhamnosus* GG suppresses the development of experimental allergic asthma in adult mice (5, 6).

Although the exact mechanism(s) behind the antiallergic action of certain bacteria remain obscure, studies have highlighted several potential components of this response. The development of mucosal and systemic tolerance relies on immunosuppressive mechanisms orchestrated by regulatory T-cell classes (Treg cells) that attenuate both Th1 and Th2 responses, and there is accumulating evidence linking the immunomodulatory function of microbial components and/or commensal bacteria to Treg and their associated cytokines. *In vitro,* selective bacteria have been shown to induce IL-10 producing Treg (8). Whereas *in vivo*, treatment with a preparation of heat-killed *Mycobacterium vaccae* can induce allergen-specific Treg ($CD4\ CD45Rb^{lo}\ IL\text{-}10^+$) that confer protection against allergic airway inflammation (9). Furthermore, Feleszko and colleagues recently demonstrated that early-life treatment with *L. rhamnosus* GG leads to an attenuated allergic airway response in adult animals that is associated with an increased T-cell expression of Foxp3, a key transcription factor in programming $CD4^+CD25^+$ Treg (5), whereas *Bifidobacterium infantis* induces $Foxp3^+$ T cells that protect mice against *Salmonella typhimurium* infection (10). Given the association between certain microorganisms and Treg, together with observations made in experimental asthma models that Treg can suppress allergen-induced airway eosinophillia and hyperresponsiveness (9–13), it was the aim of this study to investigate the potential role of regulatory T cells in mediating *L. reuteri*–induced attenuation of allergic

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airway inflammation. Portions of the data presented in this article have been previously published in abstract form (11).

MATERIALS AND METHODS

Bacterial Preparation

L. reuteri were purchased originally from the American Type Culture Collection (ATCC #23272). *L. salivarius* were a gift from Dr. B. Kiely (Alimentary Health, Cork, Ireland). Both strains are of human intestinal origin. Both strains were prepared from frozen stocks (−80°C) as described previously (7).

Animals

Adult male BALB/c mice (20–25 g) were maintained in an automatic light/dark cycle (light periods of 12 hours) and provided water and chow ad libitum. Mice were acclimatized to the animal facility for 1 week before experimentation. Age-matched (8–9 wk old) mice were used in all experiments. These experiments were performed in accordance with guidelines of the Canadian Council for Animal Care.

Bacterial Treatment

Naive mice received 1×10^9 *L. reuteri* or *L. salivarius* in 200 μL of Man-Rogosa-Sharpe liquid medium (MRS broth; Difco Laboratories, Detroit, MI) broth via a gavaging needle daily for 3, 5, or 9 days. Control animals were treated daily with 200 μL of MRS broth alone. After treatments, animals were killed and spleens removed and processed for flourescent-activated cell sorter (FACS) analysis or cell isolation.

FACS Analysis

Single cell suspensions from spleen were resuspended at 1×10^6 cells/ml and stained with extracellular CD4 and CD25 (BD Pharmingen, San Diego, CA) and intracellular IL-10 and Foxp3. For Foxp3 expression, cells were stained for surface markers and then fixed, permeabilized, and stained for Foxp3 (eBiosciences, San Diego, CA) as recommended by the manufacturer. For intracellular cytokine expression, cells (1×10^6 cells/ml) were stimulated by plate bounded anti-CD3 and soluble anti-CD28 in 96-well cell culture plates and were incubated for 6 hours in the presence of the protein transport inhibitor GolgiStop (BD Biosciences). Cells were stained for surface antigens before fixation and permeabilization and staining for intracellular cytokine expression (BD Bioscience, Mississauga, Canada). Data were acquired with LSRII or BD FACSArray (Becton Dickinson, Oakville, Canada) and analyzed with the FlowJo program (TreeStar, Ashland, OR). Absolute numbers of cells were calculated by multiplying the percentage of positive staining cells in the total acquired events by the total number of cells isolated from the tissue analyzed (whole spleen, mesenteric, and mediastinal lymph nodes).

Cell Isolation

$CD4^+CD25^+$ cells were isolated from spleens of bacteria or vehicle-treated mice to 89% (±3%) purity using MACS bead mouse $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotec, Auburn, CA). $CD4^+CD25^-$ cells were isolated from the negative fraction to a purity of 94% (±2%).

Cell Activation

Ninety-six-well cell culture plates were coated with anti-CD3 (10 μg/ml) for overnight, and cells were plated at a density of 1×10^6 cells/ml with soluble anti-CD28 (2 μg/ml) for 48 hours Hereafter, supernatants were collected and stored at −20° C before cytokine quantification.

Cell-Suppression Assay

To examine the suppressive capacity of *L. reuteri*-induced T-regulatory cells, we used a well-described *in vitro* assay of T-regulatory cell activity (12). Briefly, $CD4^+CD25^+$ cells from *L. reuteri*- or vehicle-fed mice were freshly isolated *ex vivo* to >89% purity. This cell subset was cultured with $CD4^+CD25^-$ responder T cells (50,000) for 3 days in U-bottomed 96-well plates at different ratios in the presence of soluble anti-CD3 (10 μg/ml) and soluble anti-CD28 (2 μg/ml). Responder cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) to distinguish them from T regulatory cells in co-culture. Proliferation was assayed by CFSE dilution using FACS analysis.

Experimental Asthma

This study used an OVA-sensitized mouse model of allergic airway inflammation. Briefly, mice were sensitized by intraperitoneal injection of 20 μg OVA adsorbed with 500 μg alum in saline on Day 0 and Day 6. On Days 12 and 14, mice were challenged intranasally with 5 μg OVA per mouse. Twenty-four hours after the last challenge (Day 15), mice were subjected to measurements of airway responsiveness followed by bronchoalveolar lavage (BAL). OVA/alum-sensitized, saline-challenged mice served as control animals. Where indicated, mice received 0.5×10^6 $CD4^+$ $CD25^+$ cells via tail vein injection on Day 7 of the model.

Bronchoalveolar Lavage

At 48 hours following the final OVA challenge, two aliquots of 250 μl phosphate-buffered saline (PBS) were injected and withdrawn through a tracheal cannula. Cells were removed from BAL fluid by centrifugation at $200 \times g$ for 15 min, supernatants were stored at −80°C until evaluation of cytokine content and cells were resuspended in PBS (1 ml). BAL cells were stained with Trypan blue, and viable cells counted using a hemocytometer. Smears of BAL cells were prepared with a Cytospin (Thermo Shandon, Pittsburgh, PA) and stained with HEMA 3 reagent (Biochemical Sciences, Swedesboro, NJ) for differential cell counts. A total of 200 cells were counted for each lavage, and lymphocytes, or eosinophils. An independent observer blinded to the experimental conditions performed all cell counts.

Lung Histology

Lungs were inflated with 10% formalin (to a pressure of 20 cm H_2O), fixed for 24 hours, and embedded in paraffin. Fixed and embedded tissue was stained with hematoxylin and eosin for histologic assessment using light microscopy.

Quantification of Cytokines

Cytokine levels in BAL fluid and cell culture supernatants were measured using Cytometric Bead Array Flex Sets (BD Biosciences) with the exception of transforming growth factor (TGF)-β that was quantified using an ELISA kit (R&D Systems, Inc). Both assay systems were used according to manufacturer's instructions.

Airway Responsiveness

At 24 hours after the final OVA challenge, bronchial reactivity to aerosolized methacholine was measured using whole-body plethysmograph (Buxco Electronics, Troy, NY) to determine changes in enhanced respiratory pause (Penh) (13, 14), and results were confirmed with measurement of airway resistance. For whole-body plethysmograph unrestrained, conscious mice were placed in the chambers, and after 10 minutes of stabilization, increasing concentrations of methacholine (2–32 mg/ml) were aerosolized for 3 minutes each, and mean Penh values were obtained over 5-minute periods. For measurement of airway resistance, mice were anesthetized with an intraperitoneal injection of 0.1 ml/10 g body weight of a solution of 0.4 mg/ml xylazine and 8 mg/ml ketamine in PBS. A tracheotomy was performed and an 18G tracheal cannula was inserted. Measurement of airway resistance (cm H_2O/ml/s) in response to increasing concentrations of aerosolized methacholine was obtained through the flow interrupter technique, modified for use in mice, and described in detail elsewhere (7, 15).

Statistics

Experimental results are expressed as means ± the standard errors of the means. All data were analyzed using the nonparametric Mann-Whitney test or Kruskal-Wallis test, with a Dunn multiple comparison post-hoc test (GraphPad PRISM version 5.0). A p value of less than 0.05 was considered statistically significant.

RESULTS

Lactobacillus reuteri Induces Foxp3 Expression in $CD4^+CD25^+$ Cells in the Spleen

To investigate whether oral treatment with lactobacilli leads to an increase in systemic regulatory T cells, we assessed Foxp3

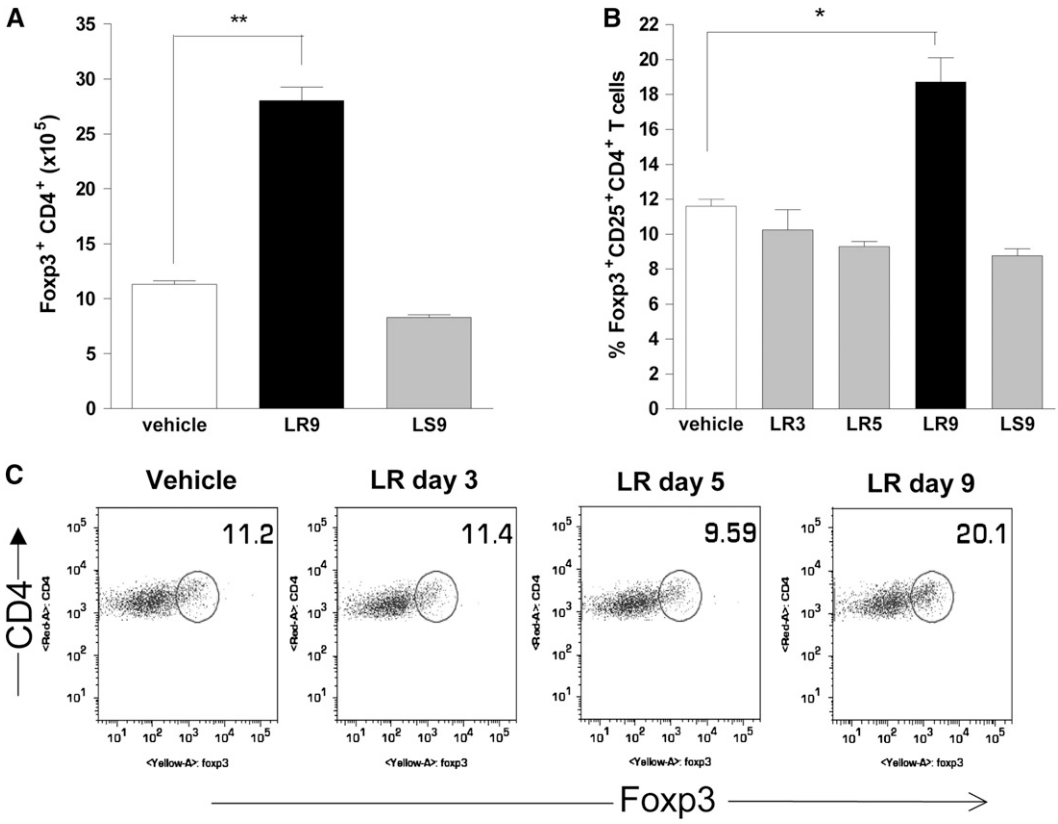

Figure 1. (*A*) Total number of Foxp3$^+$ cells in CD4$^+$ T-cell population in spleen of mice fed with *L. reuteri* (LR9), *L. salivarius* (LS9) or vehicle for 9 days. (*B*) Percentage of Foxp3$^+$ cells in CD4$^+$CD25$^+$ population in *L. reuteri* (LR9), *L. salivarius* (LS9) and vehicle-fed mice. (*C*) Representative dot plots of Foxp3$^+$ cells in the CD4$^+$CD25$^+$ population following 3-, 5-, and 9-day *L. reuteri* treatment. Data are presented as mean ± SEM (n = 10, *$P < 0.05$; **$P < 0.001$ compared with vehicle-treated mice).

expression in splenocytes following 3, 5, or 9 days of oral treatment with either *L. reuteri* or *L. salivarius*. After 9 days of treatment with *L. reuteri* the number of Foxp3 expressing CD4$^+$ cells, as determined by FACS analysis of total splenocytes, increased dramatically (Figure 1A). This was associated with a 1.6-fold increase in the percentage of Foxp3 expressing CD4$^+$CD25$^+$ cells in the *L.reuteri* compared with vehicle-treated mice (Figures 1B and 1C). Such increases in Foxp3 expression were not observed after feeding with *L. salivarius* or shorter, 3- or 5-day treatments with *L. reuteri (*Figures 1A and 1B).

We also determined that treatment with *L. reuteri* led to increased Foxp3 expression in OVA- sensitized and challenged animals. Daily treatment of OVA-sensitized mice with *L. reuteri* for 9 days before final antigen challenge led to a significant increase in Foxp3+ T cells compared with vehicle-treated animals in both the spleen ($50.0 \pm 2.9 \times 10^4$ vs. $38.2 \pm 3.4 \times 10^4$; n = 6, $P < 0.05$) and mediastinal lymph nodes ($18.6 \pm 1.6 \times 10^4$ vs. $9.1 \pm 1.5 \times 10^4$; n = 6, $P < 0.05$) (Figures 2A and 2B).

L. reuteri Treatment Up-regulates IL-10 but not TGFβ

Treatment with *L. reuteri* lead to an increase in IL-10 production by CD3/CD28 activated splenocytes (Figure 3A). Although this increase was statistically significant following 5 days of feeding (37.9 ± 1.8 pg/ml vs. 94.42 ± 18.4 pg/ml for vehicle control and 5 days of treatment group, respectively, n = 6, $P < 0.05$) there was a further, more than twofold, increase in IL-10 production between the 5-day and 9-day treatment groups. Changes in TGFβ levels in response to splenocyte activation were not observed in any of the treatment groups (Figure 3B).

To further investigate the source of increased IL-10 production in the spleen, we analyzed the effects of oral *L. reuteri* treatment on the IL-10 production of individual T cells after CD3/CD28 stimulation by assessing intracellular staining of CD4$^+$ splenocytes at the single-cell level. Treatment of mice with *L. reuteri* for 9 days led to an almost twofold increase in numbers of IL-10 producing CD4$^+$ T cells in the spleen compared with vehicle-treated mice (Figures 3C and 3D).

CD4$^+$CD25$^+$ Cells from *L. reuteri*–fed Mice Have Increased Suppressive Ability *In Vitro*

Having determined that there was an increase in Foxp3 expression in CD4$^+$ CD25$^+$ cells in the spleen from mice treated for 9 days with *L. reuteri* we wanted to assess whether

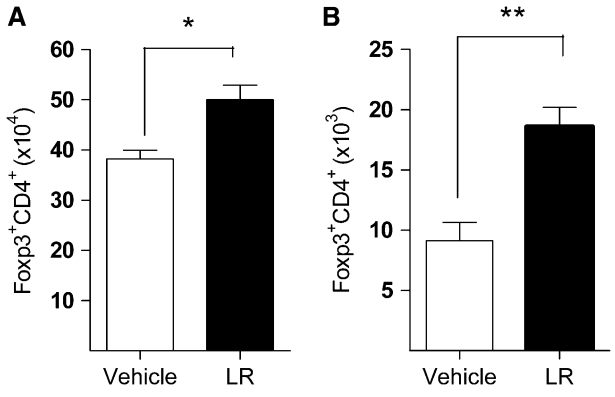

Figure 2. Effect of *L reuteri* (LR) treatment on Foxp3$^+$ T cells in ovalbumin (OVA)-sensitized and OVA-challenged mice. OVA-sensitized BALB/c mice were given LR or vehicle by gavage for 9 days before the final intranasal antigen challenge. The graphs represent the total number of Foxp3$^+$ cells in the CD4$^+$ T-cell population in (*A*) spleen and (*B*) mediastinal lymph nodes. Data are presented as mean ± SEM (n = 6, *$P < 0.05$; **$P < 0.001$, compared with vehicle-treated mice).

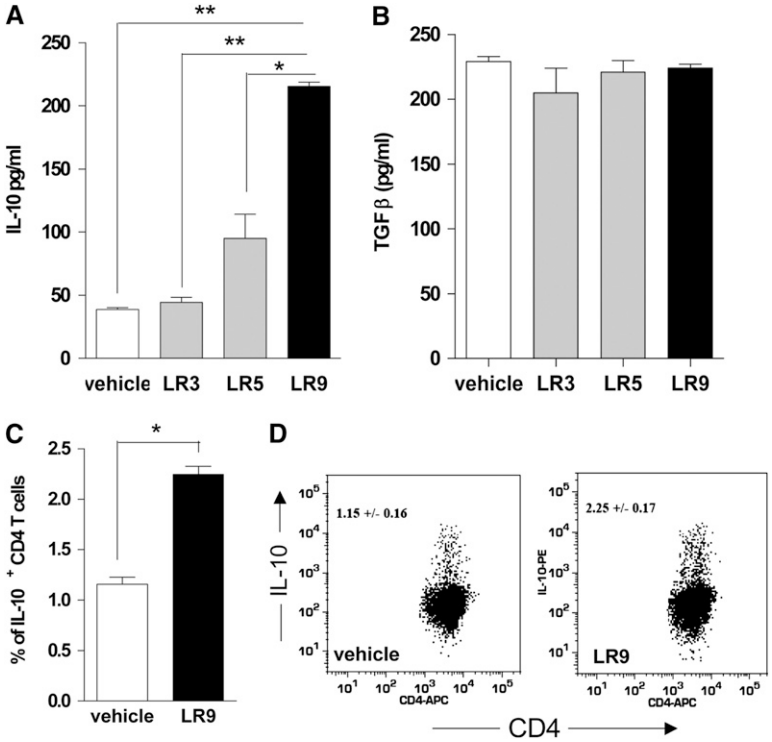

Figure 3. Effect of *L. reuteri* (LR) on IL-10 and transforming growth factor (TGF)-β expression by splenocytes. (*A*) IL-10 and (*B*) TGF-β release from CD3/CD28 stimulated splenocytes from mice fed for 3, 5, and 9 days with *L. reuteri* (LR)-fed or vehicle-fed mice. (*C*) Percentage of IL-10$^+$ cells among the CD4$^+$ T-cell population in LR-fed or vehicle-fed mice. (*D*) Dot plots, representative of six similar experiments, showing IL-10$^+$ cells among the CD4$^+$ T-cell population in LR-fed or vehicle-fed mice. Data are presented as mean ± SEM (n = 6, $*P < 0.05$; $**P < 0.001$).

this was associated with a change in the functionality of these cells. CD4$^+$CD25$^+$ cells from 9-day *Lactobacillus reuteri*– or vehicle-fed mice were freshly isolated *ex vivo*. These T regulatory cell subsets were co-cultured with CFSE-labeled CD4$^+$ CD25$^-$ T cells from naive Balb/c mice under activating conditions. Cells from *L. reuteri*–treated mice showed a significant (fourfold) increase in their ability to suppress proliferation of activated CD4$^+$CD25$^-$ cells when compared with vehicle-treated controls (Figures 4A and 4B).

CD4$^+$CD25$^+$ Cells from *L. reuteri*–fed Mice Attenuate Inflammatory Cell Influx to the Airway

Total cell numbers in BAL fluids were significantly increased 48 hours after the final antigen challenge in OVA-sensitized

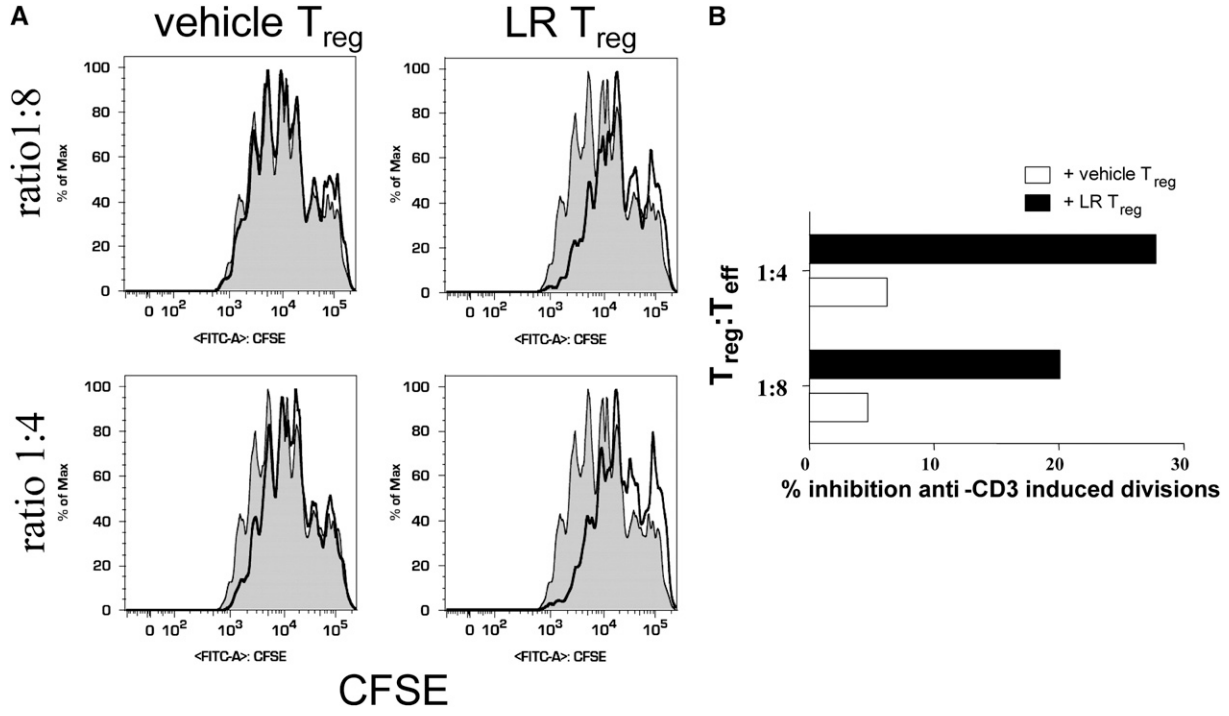

Figure 4. Effect of *L. reuteri* (LR) treatment on the *in vitro* suppressive capacity of splenic CD4$^+$CD25$^+$ cells. (*A*) CFSE plots, representative from 3 similar experiments, of the T effector–cell proliferation in the absence (*tinted histograms*), and presence (*open histogram*), of CD4$^+$CD25$^+$. (*B*) Percentage of inhibition of effector T-cell proliferation by CD4$^+$CD25$^+$ from LR-fed and vehicle-fed controls. CFSE = carboxyfluorescein diacetate succinimidyl ester.

mice than in OVA-sensitized saline-challenged mice (89.4 ± 17.9 × 10^4 vs. 7.2 ± 0.8 × 10^4, respectively; n = 10, $P < 0.01$), thus confirming that the challenge with OVA was effective. Although the cell population in BAL fluid from saline-challenge mice was almost exclusively alveolar macrophages, the OVA challenge caused a dramatic increase in the proportion of eosinophils (Figure 5A). OVA-challenged mice receiving $CD4^+$ $CD25^+$ cells from non–OVA-sensitized mice treated with *L. reuteri* for 9 days had a significant reduction in total cells recovered in BAL fluid compared with those receiving cells from vehicle–control treated mice (26.2 ± 4.9 × 10^4 vs. 89.4 ± 17.4 × 10^4, respectively; n = 10, $P < 0.01$). This corresponded with a marked decrease in eosinophils (15.4 ± 2.9 × 10^4 vs. 67.9 ± 13.5 × 10^4; n = 10, $P < 0.01$) and a smaller but significant decrease in macrophages (9.2 ± 1.7 × 10^4 vs. 22.4 ± 9.2 × 10^4; n = 10, $P < 0.05$) (Figure 5A). Histological analysis demonstrated that adoptive transfer of $CD4^+CD25^+$ cells from *L. reuteri*– but not *L. salivarius*–treated mice also attenuated the increase in eosinophils in the lung parenchyma of OVA-sensitized and OVA-challenged mice (Figure 5B).

$CD4^+CD25^+$ Cells from *L. reuteri*–fed Mice Reduce Inflammatory Cytokine Levels in BAL

Adoptive transfer of $CD4^+CD25^+$ cells from mice treated with *L. reuteri* for 9 days significantly attenuated the increase in MCP-1/CCL2, tumor necrosis factor (TNF) and IL-5 observed following OVA challenge (Table 1). Significant changes in BAL cell numbers or cytokine levels were not observed after transfer of $CD4^+CD25^+$ cells from mice treated with the probiotic for either 3 or 5 days.

$CD4^+CD25^+$ cells from *L. reuteri*–fed Mice Attenuate the Development of Airway Hyperresponsiveness

In addition to effects on airway inflammation, we wished to determine the effects of $CD4^+CD25^+$ adoptive transfer on another defining characteristic of the asthmatic response, airway hyper-responsiveness. Intranasal antigen challenge of OVA-sensitized mice led to heightened airway responsiveness as determined by increased measurements of airway resistance (Figure 6A) and Penh (Figure 6B) in response to doses of inhaled metacholine. The development of this increased airway response was significantly attenuated following adoptive transfer of $CD4^+$ $CD25^+$ cells from mice treated with *L. reuteri* for 9 days but not from mice treated with *L. salivarius* (Figure 6A). Furthermore, $CD4^+$ $CD25^+$ from mice treated with *L. reuteri* for 3 or 5 days could not attenuate the increased airway responsiveness.

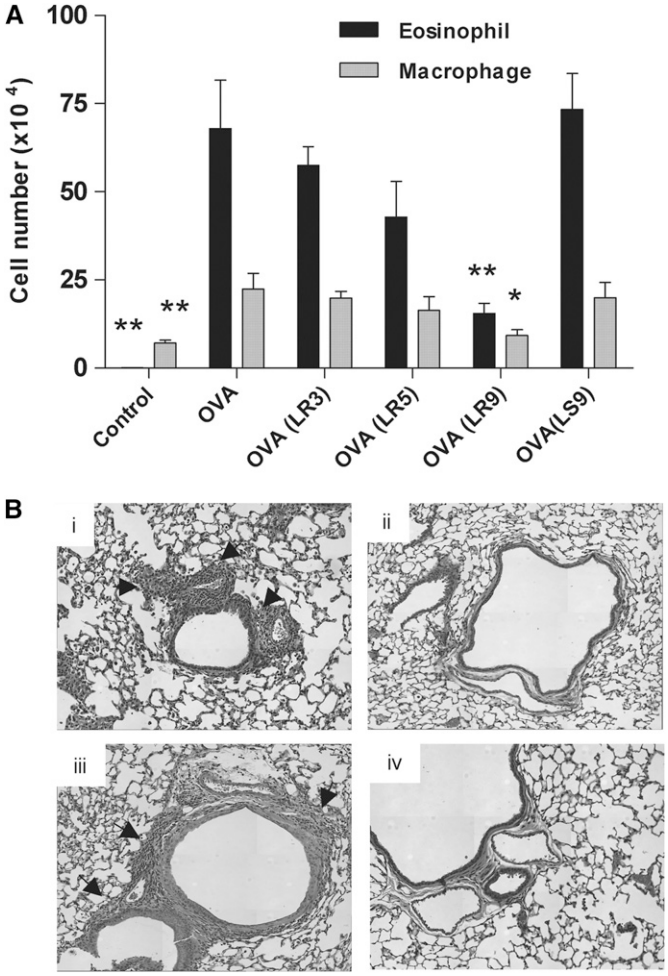

Figure 5. (*A*) Effect of adoptive transfer of $CD4^+CD25^+$ cells from mice treated with *L. reuteri* (LR) or *L. salivarius* (LS) for 3, 5, or 9 days on absolute numbers of macrophages and eosinophils in bronchoalveolar lavage (BAL) fluid from ovalbumin (OVA)-sensitized recipient mice following intranasal challenge with OVA. (*B*) Representative sections of lung tissue following antigen challenge in OVA-sensitized mice receiving with $CD4^+CD25^+$ cells from (i) vehicle, (ii) *L. reuteri*, and (iii) *L. salivarius*–treated mice. A section from (iv) a saline-challenged control mouse is shown for comparison. Arrows indicate inflammatory cell influx to the parenchyma. $CD4^+CD25^+$ cells from *L. reuteri*–treated animals markedly reduced this influx. Data are presented as mean ± the SEM (n = 10, *$P < 0.05$; **$P < 0.001$ compared with OVA sensitized and challenged mice receiving cells from vehicle-treated mice.)

DISCUSSION

Studies indicate that diverse populations of Treg play an important role in regulating Th2 responses to allergen and maintaining functional tolerance (16). Treg cells can be detected at sites of inflammation, and in many situations, their ability to migrate to and remain in inflamed tissue is important for their function *in vivo* (16, 17). It has been demonstrated in rodent asthma models that $CD4^+CD25^+Foxp3^+$ cells are recruited into both lungs and draining lymph nodes and that Treg can suppress allergen-induced airway eosinophillia, mucous hypersecretion, and hyperresponsiveness (9, 18–22). Strickland and colleagues demonstrated that repeated cycles of T-cell activation and resultant AHR during chronic exposure of sensitized rats to aerosolized allergen were prevented by $CD4^+CD25^+Foxp3^+$ T cells with potent regulatory activity, which appear in the airway mucosa and regional lymph nodes within 24 hours of exposure (13). Although Leech and colleagues demonstrated that the natural resolution of an allergic airway response to house dust mite allergen Der p1 in mice was dependant on $CD4^+CD25^+Foxp3^+$ cells that appear in lungs and draining mediastinal lymph nodes after airway challenge (18).

Here we demonstrate that treatment with a specific Lactobacillus strain, shown in our previous study to have antiallergic effects (7), significantly increases the proportion of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the spleen of non-sensitized adult mice. In OVA-sensitized and OVA-challenged mice treated with *L. reuteri*, this increase in Foxp3 was also observed in the mediastinal lymph nodes, indicating that the induced Treg cells can migrate to the airways. Of more physiological relevance than an increase in numbers of $Foxp3^+$ T cells, *L. reuteri* treatment also leads to an enhancement in the regulatory function of $CD4^+CD25^+$ cells. *In vitro* this corresponds to an increase in the ability of $CD4^+CD25^+$ cells to

TABLE 1. CYTOKINE MEASUREMENTS IN BRONCHOALVEOLAR LAVAGE

	Challenge/Treatment*					
Cytokine (*pg/ml*)	Saline	OVA	OVA/LR3	OVA/LR5	OVA/LR9	OVA/LS9
TNF	2.32 ± 1.4**	36.7 ± 8.8	34.6 ± 7.4	25.6 ± 8.6	14.4 ± 4.2†	35.4 ± 7.4
MCP-1 /CCL2	n.d.	72.8 ± 18.7	68.8 ± 11.3	58.6 ± 10.4	23.7 ± 7.1†	62.5 ± 11.2
IL-5	n.d.	189.5 ± 10.6	162.2 ± 12.1	149.4 ± 14.4	68.4 ± 13.6†	157.2 ± 11.6
IL-10	22.6 ± 2.5	16.8 ± 1.6	18.9 ± 1.8	22.2 ± 1.4	26.4 ± 2.6	24.3 ± 1.5

Definition of abbreviations: CCL2 = chemokine (C-C motif) ligand 2; LR = *Lactobacilus* reuteri; LS = *Lactobacilus* salivarious; MCP-1 = monocyte chemotactic protein-1; n.d. = not detectable; OVA = ovalbumin; TNF = tumor necrosis factor.

* The effect of adoptive transfer of $CD4^+CD25^+$ cells from mice fed with *L. reuteri* or *L. salivarius* for 3, 5, or 9 days on cytokine levels in bronchoalveolar lavage fluid of OVA-sensitized and OVA-challenged mice. Values are mean ± SEM; n = 10. Saline-challenged negative control values are also given.

** $P < 0.05$.

† $P < 0.001$.

suppress proliferation of effector T cells ($CD4^+CD25^-$). Perhaps most significantly, adoptive transfer of these $CD4^+CD25^+$ cells to OVA-sensitized mice results in attenuation of airway inflammation and hyperresponsiveness after inhaled antigen challenge. This reduction in allergic airway response is similar in characteristics and magnitude to that described previously after a direct feeding of *L. reuteri* to OVA-sensitized mice (7). Although Feleszko and colleagues (5) recently demonstrated that *L. rhamnosus* GG-induced attenuation of allergic responses in a mouse model of asthma was associated with an increase in Foxp3 expression by T cells, our current study is the first to provide evidence of a functional link between treatment with specific commensal bacteria, increased regulatory T cells activity, and attenuation of the allergic airway response.

Zuany-Amorim and colleagues reported that heat-killed *Mycobacterium vaccae* suppress antigen-induced eosinophilic airway inflammation by induction of antigen-specific $CD4^+CD45RB^{low}$ Treg that confer this protective effect in an adoptive transfer model (9). In contrast, the regulatory response induced by *L. reuteri* does not require prior exposure of Treg to the specific allergen, a response similar to that observed after infection with certain gastrointestinal helminths (22–25). For example, Wilson and colleagues demonstrated that $CD4^+CD25^+$ T cells from the mesenteric lymph nodes of *H. polygyrus*-infected, allergen-naive mice were able to confer suppression of an allergic airway response when transferred to uninfected, allergen-sensitized recipients (25).

Although the suppressive activity of Treg cells requires prior activation through their T-cell receptor, once activated, Treg cells can suppress in an antigen-nonspecific way called "bystander suppression." Thus, Treg cells with one antigen specificity can suppress effector T cells with many other distinct antigen specificities (17, 25). In addition, it is known from *in vivo* transfer studies that a population of Treg cells can create a regulatory milieu that promotes the outgrowth of new populations of Treg cells with antigen specificities distinct from those of the original Treg population (17, 26, 27). In this way *L. reuteri* may induce Treg mediated suppression that can spread beyond the local tissue and exert long-lasting influence over the immune system.

It is recognized that there are clear strain-specific immunoregulatory properties of *Lactobacillus* and *Bifidobacterium* species both *in vivo* and *in vitro* and the probiotic effects of a given strain cannot be extrapolated to other strains (8, 28–32). Smits and colleagues (8) observed that certain *Lactobacillus* species, but not others, could induce dendritic cells to drive the formation of IL-10 producing Treg cells that were capable of inhibiting the proliferation of bystander T cells. Whereas Foligne and colleagues (28) demonstated that mouse bone marrow–derived dendritic cells, pulsed with selected *Lactobacillus* strains, could attenuate trinitrobenzene sulphonic acid (TNBS)-induced colitis via a $CD4^+CD25^+$ cell-dependent mechanism. Even strains within the same *Bifidobacterium longum* species have been shown to have distict immunologic properties (29). Here again, we demonstrate this phenomenon, as *L. salivarius* did not induce Foxp3 expression in $CD4^+$ cells when fed to

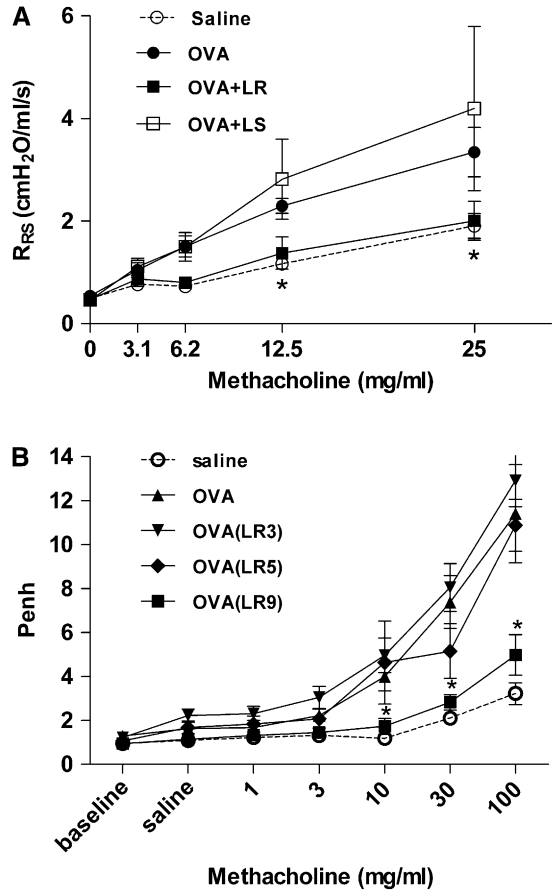

Figure 6. (*A*) Effect of adoptive transfer of $CD4^+CD25^+$ cells from mice treated with *L. reuteri* (LR) or *L. salivarius* (LS) for 9 days on airway responsiveness to methacholine, as assessed by changes in airway resistance in ovalbumin (OVA)-sensitized recipient mice 24 hours after intranasal challenge with OVA or saline (n = 5). (*B*)The effect of adoptive transfer of $CD4^+CD25^+$ cells from mice fed with *L. reuteri* (LR) for 3, 5, or 9 days on airway responsiveness to methacholine, as assessed by changes in Penh (n = 10). Data are presented as mean ± SEM (*$P < 0.05$ compared with OVA-sensitized and OVA-challenged mice receiving cells from vehicle-treated mice).

mice. This corresponds with our previous observation that this strain, in contrast to *L. reuteri*, does not attenuate the allergic airway response in mice (7). Although the determinants of these distinct immunologic properties are obscure, there is some evidence that the composition of cell surface molecules may determine if and how bacteria confer specific health benefits (33, 34). For example, Grangette and colleagues demonstrated that the composition of lipotechoic acid expressed on *L. plantarum* determined whether the organism modulated proinflammatory or antiinflammatory immune responses (33). Furthermore, Mazmanian and colleagues recently reported that polysaccharide A, derived from *Bacteroides fragilis* protects animals from experimental colitis through a functional requirement for IL-10-producing $CD4^+$ T cells (34). Identifying the key component(s) of *L. reuteri* that impart the ability to enhance Treg responses may have important therapeutic implications and will be the focus of future studies.

Two recent studies found preventive effects of *L. rhamnosus* GG on allergic airway responses in mice only when the bacteria were administered in the neonatal period (5, 6). This led to the suggestion that probiotic intervention might be successful only in the initial stage of colonization, a process which is believed to be crucial for the maturation of the immune system to a balanced phenotype. However, our current results, and previous studies by ourselves and others (7, 35–39), suggest that at least certain strains of lactic acid bacteria have profound immunoregulatory and antiallergic effects in the adult murine model and argue against the existence of a postulated essential early life "window of opportunity" for probiotic therapy.

In summary, these studies provide evidence that *L. reuteri*–induced attenuation of the allergic airway response in mice is mediated in a nonantigen-specific manner by $CD4^+$ $CD25^+Foxp3^+$ regulatory T cells. Although additional regulatory cell populations may play a role in mediating these effects, the magnitude of attenuation of the allergic airway response induced by transfer of $CD4^+CD25^+$ cell from *L. reuteri*–treated mice suggests a major contribution from these cells.

Our results further support the concept that oral treatment with microorganisms can exert major immunomodulatory activities that are not confined to the gastrointestinal tract. The induction of these regulatory responses suggests that selected bacteria may have beneficial effects on a range of atopic diseases. Although the current study provides insight into the mechanisms behind the antiallergic effects of a specific bacterial strain, further studies are required to identify specific components of bacteria–host interaction that determine the ability to confer these potentially beneficial immune responses. Such increased knowledge will allow us to move away from a largely empirical approach to probiotic therapy and may aid in the design of clinically effective, bacteria-based strategies for the treatment of asthma.

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