

Selective probiotic bacteria induce IL-10–producing regulatory T cells *in vitro* by modulating dendritic cell function through dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin

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Background: Lactobacilli are probiotic bacteria that are frequently tested in the management of allergic diseases or gastroenteritis. It is hypothesized that these probiotics have immunoregulatory properties and promote mucosal tolerance, which is in part mediated by regulatory T cells (Treg cells). On the basis of pathogenic or tissue-specific priming, dendritic cells (DC) acquire different T cell–instructive signals and drive the differentiation of naive T_H cells into either T_H1, T_H2, or regulatory effector T cells.

Objective: We studied in what way different species of lactobacilli prime human DCs for their ability to drive Treg cells.

Methods: Human monocyte-derived DCs were cultured *in vitro* with lactobacilli of different species.

Results: Two different species of lactobacilli, *Lactobacillus reuteri* and *Lactobacillus casei*, but not *Lactobacillus plantarum*, prime monocyte-derived DCs to drive the development of Treg cells. These Treg cells produced increased levels of IL-10 and were capable of inhibiting the proliferation of bystander T cells in an IL-10–dependent fashion. Strikingly, both *L reuteri* and *L casei*, but not *L plantarum*, bind the C-type lectin DC-specific intercellular adhesion molecule 3–grabbing non-integrin (DC-SIGN). Blocking antibodies to DC-SIGN inhibited the induction of the Treg cells by these probiotic bacteria, stressing that ligation of DC-SIGN can actively prime DCs to induce Treg cells. **Conclusions:** The targeting of DC-SIGN by certain probiotic bacteria might explain their beneficial effect in the treatment of a number of inflammatory diseases, including atopic dermatitis and Crohn's disease. (J Allergy Clin Immunol 2005;115:1260–7.)

Key words: Human, dendritic cells, toll-like receptor, dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin, bacteria, IL-10

The prevalence of childhood allergy and asthma has alarmingly increased during the past few decades in westernized countries. At present, the most popular explanation for this increase is found in the hygiene hypothesis, which states that changes in the type and duration of microbial exposure during a critical time window early in life have increased the risk of allergic diseases during childhood. Epidemiologic studies point at a correlation with a changed microbial exposure at mucosal surfaces of the gut–intestinal tract. Examples of microbes that might harbor a protective effect are certain helminths (eg, *Schistosoma mansoni*), orofecal microorganisms (eg, *Toxoplasma gondii*, *Helicobacter pylori*, and hepatitis A virus), and the stable colonization of certain gram-positive gut flora bacteria, such as lactobacilli and bifidobacteria.^{1,2}

It is hypothesized that these microorganisms educate the neonatal immune system by converting the T_H2-biased prenatal responses into balanced immune responses, including tolerance to self-antigens or harmless ubiquitous antigens. Currently, the hygiene hypothesis is believed to be based on decreased tolerance mediated by otherwise microbe-induced regulatory T cells (Treg cells).³ Treg cells mediate tolerance by inhibiting the proliferation and cytokine production of other immune cells, particularly effector T cells.⁴ Therefore a lot of scientific interest has focused on developing strategies to promote the development of Treg cells through the use of specific microbial adjuvants to stop the allergic march.

Dendritic cells (DCs) are able to discriminate between different pathogenic compounds through the expression of various pattern-recognition receptors (PRRs), recognizing specific pathogen-associated molecular patterns. Examples are the families of the toll-like receptors (TLRs) and the C-type lectins,⁵ such as dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) and mannose receptor (MR), that recognize carbohydrate structures on pathogens and self-glycoproteins. On activation by microbial products, inflammatory

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Abbreviations used

cfu:	Colony-forming units
DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
MF:	Maturation factor
MR:	Mannose receptor
PRR:	Pattern-recognition receptor
TLR:	Toll-like receptor
Treg cell:	Regulatory T cell

products, or both, DCs will acquire signals that drive the development of either T_H1 , T_H2 , Treg, or unpolarized T_H cell responses.⁶ The nature of these T cell-polarizing signals is largely determined by the type of microbial products, inflammatory products, or both encountered in the peripheral tissues during the immature phase. DCs primed with viruses or intracellular bacteria often drive the development of protective T_H1 cells, whereas helminth infections result in instruction for T_H2 development.^{7,8} Certain pathogens (eg, *Plasmodium falciparum*,⁹ *Bordetella pertussis*,¹⁰ *Schistosoma mansoni*,¹¹ *Mycobacteria tuberculosis*,¹² or hepatitis C virus¹³) might evade immunity-expressing molecules that can also prime DCs (regulatory DCs) for the capacity to drive the development of Treg cells. Furthermore, under homeostatic conditions, nonpathogenic gut flora microorganisms (eg, lactobacilli, lactococci, or bifidobacteria) might harbor similar qualities, which then will be fruitful in the prevention of excessive inflammation, allergy, or autoimmune diseases.

An important example of such microorganisms are probiotic bacteria, defined as live microbial food ingredients that are beneficial to health in general and that are also commensal bacteria of healthy human gut microflora. One of the most frequently used genera is *Lactobacillus*, which, importantly, is also associated with the management of allergic diseases¹⁴ and the improvement of various gastrointestinal diseases.¹⁵ It was shown earlier that different species of lactobacilli can modulate DC or T-cell responses (eg, by inducing the immunosuppressive cytokines IL-10 and TGF- β or reducing T-cell proliferation).¹⁶⁻¹⁹ To understand their working mechanism and their putative capacity as a Treg cell-inducing adjuvant in the treatment or prevention of allergic diseases, we have focused on the immunoregulatory capacity of a few lactobacilli, randomly selected from the probiotic literature, to prime for regulatory DCs as a mechanism to prevent allergic diseases.

METHODS

Antibodies, cytokines, and reagents

Human recombinant (r)IL-4 (sp. act. 1×10^8 U/mg) was obtained from PBH (Hanover, Germany). Human rGM-CSF (specific activity 1.11×10^7 U/mg) was a gift from Schering-Plough (Uden, The Netherlands). The generation of DC-SIGN antibodies (AZN-D1 and AZN-D2) was described before.¹² FLAG-tagged human TLR1 and

TLR2 were provided by Tularic (San Francisco, Calif), FLAG-tagged TLR7 in pFLAG-CMV1 was a gift from D. Golenbock (University of Massachusetts Medical School, Worcester, Mass), and FLAG-tagged TLR9 was a gift from S. Akira (Osaka University, Japan). Neutralizing antibodies to human IL-10 were obtained from BD Pharmingen (San Diego, Calif).

Bacterial strains and inoculum preparation

Lactobacillus plantarum strain NIZO B253, *Lactobacillus casei* strain NIZO B255 (both from NIZO Food Research, Ede, The Netherlands), *Lactobacillus reuteri* strain ASM20016, *Escherichia coli* strain AMC B12G1, and *Staphylococcus aureus* 4D2 were cultured on Columbia agar (Oxoid, Basingstoke, United Kingdom) containing 6.25% sheep blood. Lactobacilli were incubated at 37°C in a 5% CO₂ atmosphere, and *E coli* was incubated at room temperature under aerobic conditions. After 3 days, the number of bacteria was determined by measuring the optical density at 620 nm (OD₆₂₀). Typically, an OD₆₂₀ of 0.35 corresponded to 1×10^8 colony-forming units (cfu) for all test strains.

Monocyte-derived DCs

Immature DCs were generated from monocytes, as previously described.²⁰ On day 6, maturation of immature DCs was induced by LPS (*E coli*; Sigma-Aldrich, St Louis, Mo), different concentrations of lactobacilli, or *E coli* in the presence of the combination of the cytokines IL-1 β (25 ng/mL) and TNF- α (50 ng/mL), together named as maturation factors (MFs; both purchased from Peprotech, Rocky Hill, NJ). IL-12, IL-10, and IL-6 cytokine production by mature DCs was determined by means of a 24-hour stimulation with CD40 ligand-expressing mouse plasmacytoma cells (J558), as described elsewhere.²⁰

T_H cells

CD45RA⁺CD45RO⁻ naive CD4⁺ T cells were isolated from PBMCs through negative selection with CD4⁺ MACS MultiSort beads (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with phycoerythrin-labeled CD45RO antibodies (Dakopatts, Glostrup, Denmark). For subsequent depletion, the anti-hapten beads of the CD4⁺ isolation kit were supplemented with anti-phycoerythrin-coupled magnetic beads. Purified naive T_H cells (2×10^4 cells) were stimulated on coculture with mature DCs (5×10^3 cells) in 200 μ L of culture medium in the presence of the superantigen *S aureus* enterotoxin B (10 pg/mL; Sigma), in 96-well flat-bottom culture plates (Costar, Cambridge, Calif). At day 5, rIL-2 (10 U/mL, Cetus Corp, Emeryville, Calif) and rIL-15 (10 ng/mL; R&D Systems, Minneapolis, Minn) were added, and the cultures were expanded for the next 7 days. To determine cytokine production by T_H cells, resting T cells were restimulated on day 12 with PMA (10 ng/mL; Sigma) and ionomycin (1 μ g/mL; Sigma) for 6 hours, the last 5 hours in the presence of Brefeldin A (10 μ g/mL; Sigma). Single-cell IL-4 and IFN- γ production was analyzed by means of intracellular flow cytometric analysis, as previously described.²¹ In parallel, T cells (1×10^5) were stimulated with anti-CD3 (1:1000 dilution of ascite fluid) and anti-CD28 (1 μ g/mL) in 96-well plates. After 72 hours, supernatants were harvested, and IL-10 content was analyzed by means of ELISA, as described elsewhere.²²

T-cell suppressor activity was determined by harvesting resting T cells on day 12 and staining these cells with 3×10^{-5} M PKH-26 (Sigma) for 5 minutes at room temperature. After thorough washing, 2.5×10^4 CD4⁺ T cells (DC-primed T cells) were stimulated with anti-CD3 (1:5000 dilution of ascite fluid) and anti-CD28 (0.5 μ g/mL) in round-bottom 96-well plates. After overnight preactivation, 2.5×10^4 5,6-carboxy fluorescein diacetate succinimidyl ester-labeled peripheral CD4⁺ T cells were added, representing the responder T cells (CFSE, 0.5 μ M; Molecular Probes, Inc, Eugene, Ore). After 5 days,

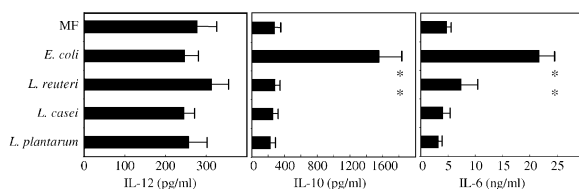


FIG 1. Cytokine production by lactobacilli-treated DCs. DC maturation was induced by the addition of MF (rIL-1 β and rTNF- α) or MF plus 10^7 cfu of bacteria. After 48 hours, mature DCs were restimulated for 24 hours with CD40 ligand-transfected J558 cells to induce the production of IL-12, IL-10, and IL-6.

the content of PKH and CFSE in the DC-primed and responder T cells, respectively, was analyzed by means of flow cytometry, and the proliferation index was determined with Modfit (BD Pharmingen).

Stimulation of HEK cells transiently transfected with TLR constructs

HEK-293 cells were transfected with TLR-expressing plasmids and an ELAM-luciferase reporter construct, as described previously.¹¹ After 24 hours, cells were stimulated with TNF- α (100 ng/mL; Strathmann Biotech, Hamburg, Germany), LPS (100 ng/mL, Sigma), PAM-3-CysSerLys (20 μ g/mL; EMC Microcollections, Tuebingen, Germany), resiquimod (R848, 10 ng/mL; InvivoGen, San Diego, Calif), CpG-oligonucleotide 2006 (10 μ M; Biosource International Nivelles, Belgium), poly I:C (20 μ g/mL, Sigma), or bacteria (10^7 /mL). After 6 hours, cells were lysed in reporter lysis buffer (Promega, Madison, Wis), and luciferase activity of the cellular lysate was measured with an assay kit from Promega according to the manufacturer's descriptions.

DC or K562-cell binding by bacteria

Bacteria (10^9 /mL) were labeled by means of incubation with FITC (0.5 mg/mL) in PBS for 1 hour at room temperature. The FITC-labeled bacteria were washed 3 times to remove unbound FITC. Immature DCs, K-562 cells, or K-562-DC-SIGN cells (5×10^4) were preincubated with neutralizing antibodies to DC-SIGN (AZN-D1 and AZN-D2), mannosylated BSA (50 μ g/mL, Sigma), mannan (50 μ g/mL, Sigma), or EDTA (10 mM) in TSM buffer (20 mM Tris, 150 mM NaCl, 1 mM CaCl₂, and 2 mM MgCl₂, pH 8.0) plus 0.5% BSA for 10 minutes at room temperature. Thereafter, the FITC-labeled bacteria (bacteria/cell ratio of 10:1) were added and incubated for 45 minutes at 37°C. After washing, the cells were analyzed by means of flow cytometry and gated for large cells in the forward scatter/side scatter, excluding unbound bacteria in the analysis. K-562 cells and K-562-DC-SIGN cells were cultured as previously described.¹²

Statistics

Data are expressed as means $\pm$ SEM. Data were analyzed for statistical significance with GraphPad InStat software (version 3.00; GraphPad InStat, Inc, San Diego, Calif) by using ANOVA, followed by the Bonferroni multiple comparison test or an unpaired Student *t* test to compare either different groups of multiple data sets or just 2 sets of data or variables between 2 groups, respectively. A *P* value of less than .05 was considered to be significant.

RESULTS

Cytokine production by lactobacilli-treated DCs

The 3 different lactobacillus species, *L. reuteri*, *L. casei*, and *L. plantarum*, were randomly selected from the many

candidate probiotics for a comparative study regarding their immunoregulatory effect on DC function, with *E. coli*, a gram-negative gut flora commensal, used for comparison. The CD40 ligand-induced production of IL-12, IL-10, or IL-6 in DCs matured through stimulation by the combination of IL-1 β and TNF- α (MFs) was not significantly affected by the presence of lactobacilli during maturation (Fig 1). In contrast, *E. coli* primed for a strong IL-10 and IL-6 production, as described earlier.²¹

L. reuteri and *L. casei* induce Treg cell development through modulation of DC function

The immunoregulatory potential of the different lactobacilli on the T_H-cell polarizing capacity of DCs was investigated by stimulating naive T cells with the superantigen *S. aureus* enterotoxin B plus DCs matured in the presence or absence of lactobacilli or *E. coli*. After 12 days, in these effector T_H cells, the intracellular profile of the T_H1 cytokine IFN- γ and the T_H2 cytokine IL-4 was determined. Our previous studies have shown that priming of immature human monocyte-derived DCs by MFs or LPS does not impose a bias in their T cell-polarizing capacity and merely results in the development of a mixed population of IL-4- and IFN- γ -producing effector T cells.^{7,20} In line with our previous study,²¹ the cytokine profile of the T cells stimulated with lactobacilli-primed DCs was similar to the cytokine profile of effector T_H cells stimulated with control DCs matured by MFs (Fig 2, A). Instead, effector T cells generated in response to *L. reuteri*- or *L. casei*-treated mature DCs produced increased levels of IL-10 (Fig 2, B; MF-control 3.56 ± 0.25 ng/mL). The various effector T-cell populations were subsequently tested for their regulatory function in a cell-cycle tracking dye-based proliferation assay. These T cells (DC-primed T cells) were labeled with red PKH-26 and cocultured for 5 days with peripheral CD4⁺ T cells (responder T cells) labeled with the green CFSE in the presence of anti-CD3 and anti-CD28. The proliferation (Fig 2, C) and proliferation index (analyzed with Modfit, BD Pharmingen) (Fig 2, D) was substantially lower in responder cells cocultured with effector T cells generated in response to *L. reuteri*- or *L. casei*-primed mature DCs in comparison with that seen in responder cells cocultured with effector T cells generated in coculture with mature DCs primed with *L. plantarum* or *E. coli* (Fig 2, C). When different doses (10^3 , 10^5 , and 10^7 cfu) of bacteria were evaluated, a maximal inhibitory effect was found for 10^5 cfu of *L. reuteri* and *L. casei* bacteria. Neither *E. coli* nor *L. plantarum* mature DC-primed T cells inhibited responder T-cell proliferation independent of the bacterial dose (Fig 2, D; data for 10^3 and 10^7 cfu of bacteria not shown). Importantly, experiments with UV-exposed bacteria yielded identical results, indicating that the regulatory DC priming does not depend on the viability of the bacteria. Subsequent neutralization of IL-10 during the proliferation test assay resulted in restoration of

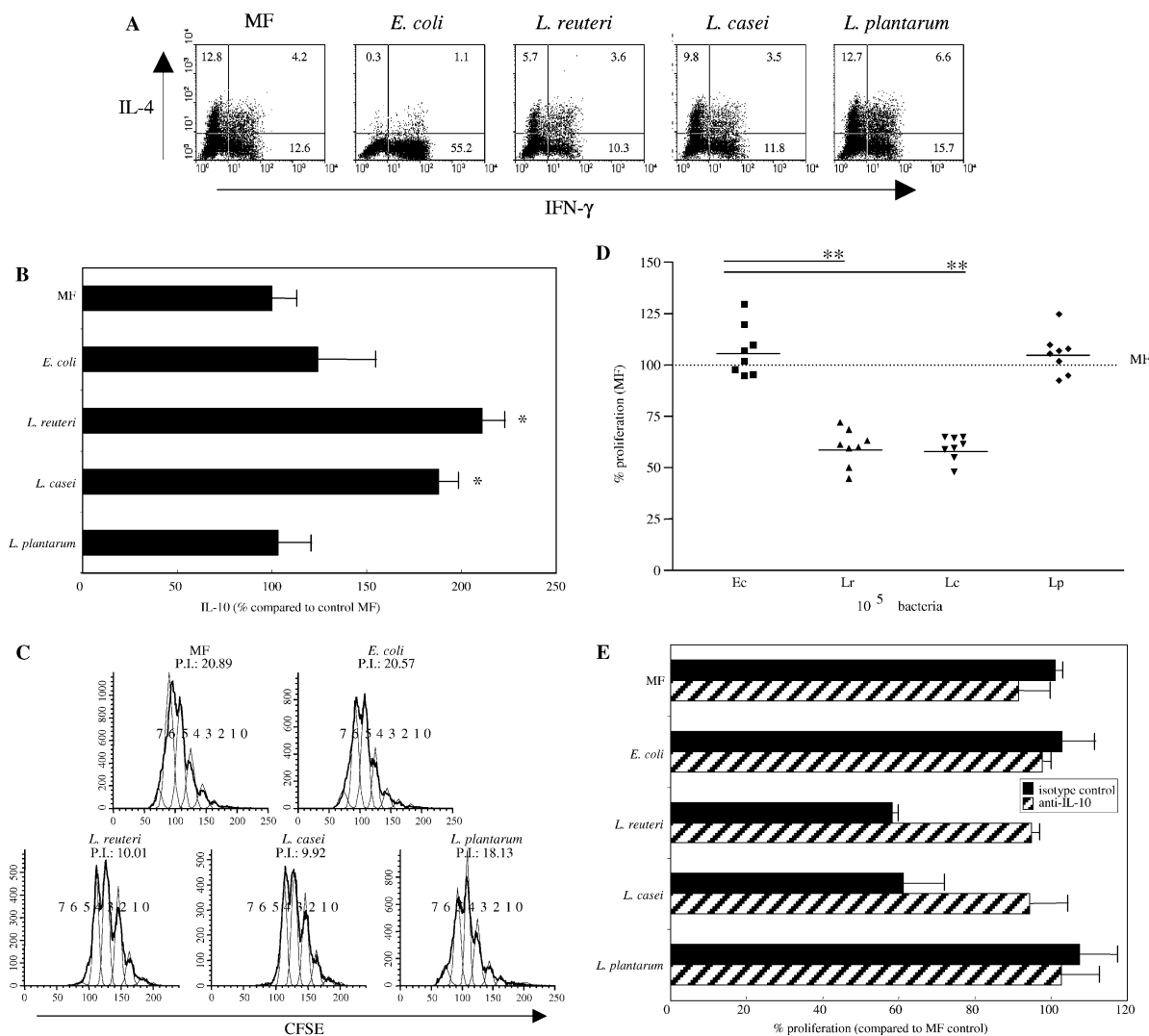


FIG 2. *L. reuteri* and *L. casei* induce Treg cell development through modulation of DC function. DC maturation was induced by the addition of MF or MF plus 10^7 cfu of bacteria (or 10^5 cfu of bacteria for Fig 2, B-D). **A**, Mature DCs were cocultured with naive T_H cells and *S aureus* enterotoxin B. After 12 days, IFN- γ and IL-4 production per cell was analyzed by means of intracellular FACS staining. **B**, Naive T_H cells were stimulated as described in Fig 2, A. After 12 days, T cells were stimulated with anti-CD3 and anti-CD28, and supernatants were collected after 72 hours, in which IL-10 levels were determined by means of ELISA. **C**, Naive T_H cells were stimulated as described in Fig 2, A. After 12 days, the DC-primed T cells were labeled with PKH-26 and stimulated with anti-CD3 and anti-CD28. After overnight incubation, CFSE-labeled responder T cells (peripheral CD4⁺ T cells) were added in a 1:1 ratio (2.5×10^4 each). After 5 days, the cells were analyzed by means of flow cytometry. **D**, The proliferation index of the CFSE-labeled responder cells cocultured in the presence of control test cells (MF) was set at 100% (absolute value in Fig 2, C), representing the maximal proliferation. * $P < .05$, ** $P < .01$. **E**, Naive T cells were stimulated as described in Fig 2, A, and tested for their suppressive capacity in the proliferation assay, as described in Fig 2, C. During the coculture of DC-primed T cells and target T cells, neutralizing IL-10 antibodies were added.

proliferation of the target T cells (Fig 2, E), indicating that the enhanced production of IL-10 by effector T cells induced by *L. reuteri*- or *L. casei*-primed mature DCs is functional for their suppressive capacity. In summary, these data indicate that *L. reuteri* and *L. casei* modulate DC function and prime for IL-10-producing Treg cells, and this process appears to be optimal under

conditions of low doses of bacteria (bacteria/DC ratio of 1:1).

TLR activation by lactobacilli

Lactobacilli, the gram-positive control bacterium *S aureus*, and gram-negative *E coli* were evaluated for their TLR-activating potential in transiently transfected

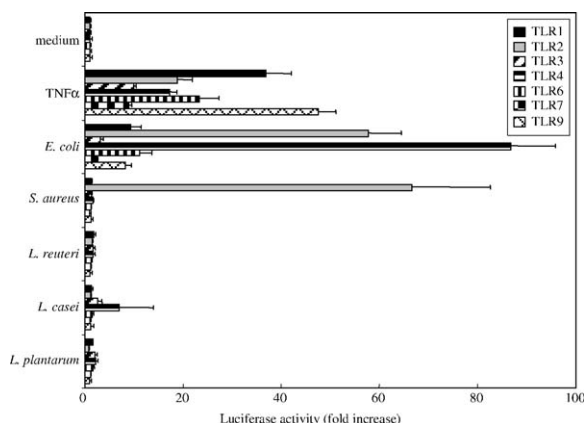


FIG 3. TLR activity by lactobacilli. HEK 293 cells were transiently transfected with human TLR1, TLR2, TLR3, TLR4 plus MD2, TLR6, TLR7, and TLR9 together with an ELAM-luciferase reporter construct and were stimulated with 10^7 cfu of bacteria or TNF- α (100 ng/mL). Luciferase activity was determined 6 hours after stimulation.

HEK-293 cells (TLR1, TLR2, TLR4 plus MD-2, TLR6, TLR7, and TLR9) by determining luciferase activity. Several positive controls were analyzed: PAM3CSK for TLR2 (fold increase, 90), LPS for TLR4 (fold increase, 47), R848 for TLR7 (fold increase, 7) and CpG for TLR9 (fold increase, 3; data not shown). *E coli* ligated TLR2 and TLR4 and TLR1, TLR6, and TLR9 to a lesser extent (probably background because *E coli* resulted in a 10-fold increase in luciferase activity in mock-transfected cells), whereas *S aureus* only ligated TLR2 (Fig 3). These data were confirmed in the stable transfectants (not shown). In contrast, the 3 lactobacilli did not activate any of the TLRs tested, except for low TLR4 activation by *L casei* at a high dose of bacteria (10^7 cfu; lower doses not shown). These results suggest that the lactobacilli tested here do not substantially activate DCs through ligation of TLRs.

L reuteri and *L casei* ligate DC-SIGN on monocyte-derived DCs

Next we evaluated which other PRRs could interact with these lactobacilli. C-type lectins are highly expressed by DCs and bind carbohydrates on pathogens in a cation-dependent manner.²³ Whereas *E coli* did not bind immature DCs, the 3 lactobacilli bound to immature DCs to different degrees. This binding was strongly inhibited by EDTA, implying a role for calcium and magnesium (Fig 4, A). In search of DC-associated molecules that might play a role in the interaction with the lactobacilli, we analyzed the binding of the bacteria to 293 T cells transiently transfected with different DC-specific C-type lectins (ie, macrophage galactose type C-type lectin, blood DC antigen 2, DC-ImmunoReceptor, Dectin-1, or DC-SIGN). Binding to DC-SIGN was alternatively tested with K-562 cells stably transfected with DC-SIGN. After subtraction of mock binding, *L casei* and *L reuteri*, the lactobacilli inducing the development of Treg cells, but not *L plantarum*, bound in variable amounts (*L casei* high and *L reuteri* low) to DC-SIGN-expressing cells (Fig 4, B). Blocking studies

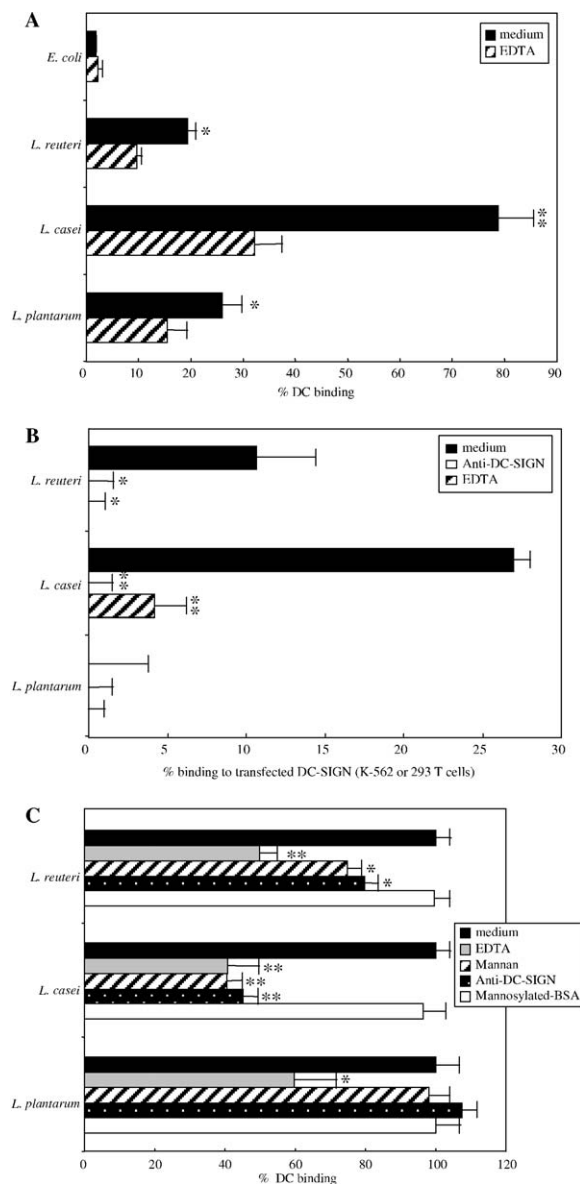


FIG 4. *L reuteri* and *L casei* ligate DC-SIGN on monocyte-derived DCs. **A**, Immature DCs were incubated with FITC-labeled bacteria (5×10^5) after a prior 10-minute preincubation with TSM buffer with or without EDTA for 45 minutes at 37°C. **B**, A similar protocol as described in Fig 4, A, was used to analyze bacteria binding to DC-SIGN-transfected K562 or untransfected K562 cells. Preincubations were performed with either TSM with EDTA or 20 μ g/mL anti-DC-SIGN (AZN-D1 and AZN-D2). **C**, DC binding was performed as described in Fig 4, A. Preincubations were performed with anti-DC-SIGN (AZN-D1 and AZN-D2), mannosylated BSA, or mannan.

demonstrated that *L reuteri* and *L casei* specifically interacted with DC-SIGN because neutralizing anti-DC-SIGN or EDTA blocked the binding completely (Fig 4, B). No binding was observed to cells expressing other C-type lectins (data not shown).

To evaluate whether DC-SIGN is the major PRR to be involved in *L reuteri* and *L casei* capture by immature DCs, we assayed the binding of lactobacilli to immature

DCs in the presence of different blocking reagents. The capturing role of DC-SIGN was substantiated by the finding that approximately half of the binding by *L casei* and a smaller proportion of the binding by *L reuteri* was abrogated by both anti-DC-SIGN and mannan, a natural ligand of DC-SIGN and MR. As expected, binding of *L plantarum* was blocked by neither anti-DC-SIGN nor mannan. The binding of lactobacilli to the MR was limited because incubation with mannosylated BSA (ligates selectively to MR) only weakly affected the binding of *L casei* to immature DCs in contrast to the other bacteria, the DC binding of which was not affected at all (Fig 4, C; absolute binding percentages are shown in Fig 4, A). In summary, these findings demonstrate that all 3 lactobacilli bind immature DCs in a cation-dependent manner, indicating that C-type lectins are involved in the capture of these bacteria. Interestingly, only the bacteria (*L reuteri* and *L casei*) that selectively prime DCs for the capacity to drive the development of Treg cells specifically ligate DC-SIGN.

DC-SIGN ligation is crucial for modulation of DC function by *L reuteri* and *L casei*

Next we investigated whether DC-SIGN is functionally involved in the priming for Treg cell development by adding blocking antibodies to DC-SIGN or mannan during the maturation of the DCs in the presence of *L reuteri* and *L casei*. Subsequently, cocultures of bacteria-primed mature DCs and naive T_H cells were performed, and the resulting effector T cells were analyzed for their capacity to inhibit responder T-cell proliferation. The blocking of ligation to DC-SIGN by *L reuteri* and *L casei* with either neutralizing antibodies or mannan abolished the priming for Treg cell development, partly in the case of *L reuteri* and almost completely in the case of *L casei*, on the basis of the restoration of the proliferation of the responder cells (Fig 5; examples of absolute proliferation index values are shown in Fig 2, C). The partial contribution of DC-SIGN in *L reuteri*-induced DC priming might imply an additional role for other PRRs. Proliferation of responder T cells cocultured with effector T cells generated in the presence of cordycepin-, *L plantarum*-, or *E coli*-primed DCs was not affected. These results demonstrate that DC-SIGN ligation by *L reuteri* and *L casei* is crucial for DCs to acquire their regulatory function.

DISCUSSION

The present study on 3 probiotic lactobacilli demonstrates that *L reuteri* and *L casei*, in contrast to *L plantarum*, prime DCs to promote the development of Treg cells. Experiments with TLR transfectants proved that all 3 lactobacilli tested did not substantially activate TLRs, except for *L casei*, which induced a low TLR4 activity at a high bacterial load. However, *L reuteri* and *L casei*, both potently inducing the development of Treg cells, were captured to different degrees by the

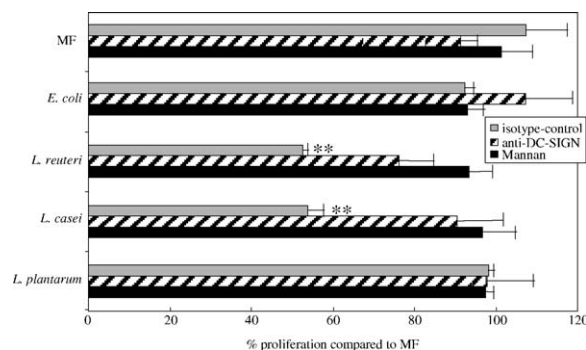


FIG 5. Blocking of DC-SIGN interaction with *L reuteri* and *L casei* abrogates the priming for Treg cell development. Immature DCs were preincubated (30 minutes at 37°C) with either isotype control antibody (20 µg/mL), anti-DC-SIGN (AZN-D1; 20 µg/mL), or mannan (50 µM). Thereafter, maturation and induction of suppressor cell activity was conducted and analyzed as described in the legend for Fig 2. **P* < .05, ***P* < .01.

PRR DC-SIGN on DCs, an interaction that appeared to be crucial for the priming of regulatory DCs.

Thus far, various microorganisms have been described to bind DC-SIGN, including *Helicobacter pylori*, mycobacteria, and its constituent ManLAM.^{12,24} Interestingly, distinct ligands show different functional effects. Ligation of DC-SIGN by mycobacteria and ManLAM inhibits LPS-induced DC maturation and induces IL-10 production,²⁵ whereas *H pylori* does not inhibit DC maturation but does induce increased levels of IL-10 in human DCs.²⁶ In sharp contrast, *L reuteri* and *L casei*, even at the high dose of 10⁷ cfu of bacteria, neither inhibit DC maturation (data not shown) nor induce increased levels of IL-10. Furthermore, DC-SIGN ligation on DCs by either specific antibodies (even in combination with type 1-inducing agents, such as poly I:C or IFN-γ; unpublished results) or by *H pylori* induces a shift toward T_H2 development,²⁶ a feature that is not shared by probiotic bacteria (as shown in this study) and ManLAM (Dr A. Engering, personal communication), which primarily prime DCs for Treg cell development. At present, it is unknown which different signals are transduced by DC-SIGN ligation.

DC-SIGN binds high mannose structures and fucose-containing Lewis antigens, which are differentially expressed in glycosylated surfaces of different pathogens.²⁴ At present, the composition and identity of carbohydrate structures on the different lactobacilli is unknown, although it was excluded that binding of *L reuteri* and *L casei* to DC-SIGN occurred through high-mannose structures or fucose residues, like Lewis x or Lewis y molecules. Differences in carbohydrate composition in the 3 lactobacilli are currently under investigation and might result in a novel carbohydrate ligand for DC-SIGN.

The lactobacilli strains used in this study did not activate any of the TLRs tested here. However, this characteristic is probably lactobacillus strain specific because ligation of TLR2, TLR4, or TLR9 by the same lactobacilli species is reported in some other studies.^{27,28}

The same holds true for their individual capacities to prime for various cytokines, such as IL-10 or IL-12, respectively.^{19,29}

Our study clearly demonstrated that optimal priming for regulatory DCs *in vitro* occurs at 10⁵ cfu of bacteria, which is a bacteria/DC ratio of 1:1, and that a significantly lower priming effect occurs at a bacteria/DC ratio of 100:1 or 10:1 (data not shown). This is an intriguing finding, suggesting that these bacteria might activate additional pathways in DCs at high doses. These pathways might interfere with the cross-talk of DC-SIGN signaling, which in the end might (partially) prevent Treg cell development. It can be speculated that this pathway, triggered at high bacterial (even probiotic bacterial) doses, represents an escape to clearance of bacterial overload, overruling the generalized immune suppression prevailing at low bacterial load. Whether different dosages of probiotic bacteria will also affect the clinical efficacy of probiotic treatment in a number of inflammatory diseases, such as allergy, remains to be established.

The regulatory function of the DC was tested in polyclonal models by using alloantigen- and superantigen-driven naive T-cell responses and not in an antigen-specific model because of the difficulty of obtaining sufficient numbers of human antigen-specific naive T cells for further analysis. Therefore the present experiments do not provide information on the antigen specificity of the induced Treg cell population. It thus remains to be established to what extent DCs primed with probiotic bacteria drive the development of Treg cells that are probiotic specific, that act as a regulatory bystander population, or both. In addition, it remains to be established how the suppression induced *in vitro* by Treg cells that are driven by the bacteria-modulated DCs described here will manifest in *in vivo* conditions. Further experiments need to be done to clarify these issues.

Probiotic bacteria are interesting candidates in the therapy of several inflammatory diseases, such as gastroenteritis.³⁰ Furthermore, a first series of clinical studies, in which high-risk children received probiotic bacteria during the first half year of life, has shown a significantly decreased incidence of atopic dermatitis until the age of 4 years.^{15,31} It is attractive to suggest that for some bacteria, these beneficial effects might, at least in part, be explained by the priming of DCs for regulatory T-cell development. However, caution must be taken because these *in vivo* effects might also be due to other factors, such as differential adherence of bacteria to epithelial cells or changes in the composition of the endogenous microflora-repelling pathogenic microorganisms. On the other hand, if the induction of Treg cells is crucial in the amelioration of disease, these therapies might be improved by the prior screening of probiotic bacteria for their potential to bind DC-SIGN and to induce Treg cells. Furthermore, if more detailed knowledge of the mechanisms underlying their effect is available, it might well be possible to improve therapy by using cocktails of different probiotics, combining specialized regulatory features of the various probiotics.

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Correction

With regard to the March 2005 editorial entitled "Pathogenesis and management of anaphylaxis: Current status and future challenges" (2005;115:571-4): Because of an error on the part of the publisher, the names of researchers appeared incorrectly in the sixth paragraph. The first three sentences of that paragraph should have appeared as follows:

Henry Metzger and his colleagues identified the high-affinity receptor for IgE as a single molecular entity (FcεRI),⁶ and Jean-Pierre Kinet and Henry cloned and characterized the genes for the 3 components of this tetrameric receptor.^{6,7} Through the work of Henry, Jean-Pierre, and many others, it is now known that the aggregation of FcεRI that occurs in anaphylaxis and other clinical settings by the binding of FcεRI-bound IgE to bivalent or multivalent antigen induces the activation of mast cells and basophils, resulting in the rapid and, in the case of cytokines and chemokines, sustained release of diverse mediators, many of which can induce the pathophysiologic components of anaphylaxis.⁶⁻⁸