Instruction of effector T cell programs by flexible dendritic cells
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Selected probiotic bacteria induce regulatory T cells by modulating dendritic cell function via DC-SIGN


Abstract
Lactobacilli are one of the most frequently used probiotic bacteria in the management of gastroenteritis or allergic diseases. It is hypothesized that these probiotic bacteria have immunoregulatory properties and promote mucosal tolerance. This form of negative regulation of adaptive immunity is in part mediated by regulatory T cells generated from naïve T cells. Based on pathogenic or tissue-specific priming, dendritic cells (DC) develop different instructive signals and drive the differentiation of naïve T helper (Th) cells into either Th1, Th2 or regulatory effector T cells. In this study, we demonstrate that two different species of lactobacilli, lactobacillus reuteri and lactobacillus casei, but not lactobacillus plantarum, prime human monocyte-derived DC (moDC) to drive the development of regulatory T cells, capable of inhibiting the proliferation of bystander T cells. None of the three lactobacillus species tested, induced full DC maturation, which was paralleled by an inability to activate TLR1, 2, 4, 6, 7 and 9. Strikingly, both L. reuteri and L. casei and not L. plantarum, bind the C-type lectin DC-specific ICAM-3-grabbing non-integrin (DC-SIGN). Blocking antibodies to DC-SIGN inhibited the induction of the regulatory T cells. These findings suggest that selected probiotic bacteria can prime DC for a negative regulatory potential via interaction with DC-SIGN, and this may explain their beneficial effect in the treatment of a number of inflammatory diseases, including Crohn’s disease and atopic dermatitis.
Introduction

Probiotics are defined as live microbial food ingredients that are beneficial to health and they are also commensal bacteria of healthy human gut microflora. One of the most frequently used genera is lactobacillus, which is associated with the improvement of various gastrointestinal diseases (1,2) and the management of allergic diseases (3,4). The effects of probiotics can be either direct through modulation of the endogenous microflora composition or indirect through modulation of the immune system, in part by influencing the balance between pro- and anti-inflammatory events. For example, studies with germ-free animals have demonstrated that the gutflora is crucial for the development of oral tolerance to innocuous ingested proteins or to nonpathogenic microbial compounds (5). To date, no specific gutflora bacterial species, uniquely involved in these processes, have been identified. Lactobacillus species may form potential candidates, as they show immunoregulatory capacities in vitro and in vivo (6,7).

Mucosal tolerance is mediated by specialized regulatory T cells, inhibiting the proliferation and cytokine production of other immune cells, in particular that of effector T cells (8). The importance of regulatory T cells has been demonstrated indifferent murine models of colitis, including a model in which co-transfer of CD4+CD45RBlo T cells together with pathogenic CD4+CD45RBghi T cells into syngeneic SCID or RAG−/− recipients prevents disease, due to the presence of regulatory T cells (9,10). Part of this regulatory potential was enriched in the T cell population expressing CD25 and representing the thymic-derived naturally occurring regulatory T cells (11). However, control of immune pathology was not completely restricted to CD4+CD25+ T cells, as there is evidence that also CD4+CD25− T cells harbor regulatory activity (11,12). Studies with blocking antibodies to TGFβ or IL-10R, or studies with IL-10 deficient mice showed that CD4+CD45RBlo cells were no longer able to inhibit colitis, implying that IL-10 and/or TGFβ are instrumental for their inhibitory capacity (9,10). These IL-10 and/or TGFβ-producing T cells, so-called Tr1 and/or Th3 cells (13), can also be generated in vitro from peripheral CD4+ T cells by repetitive stimulation in IL-10 (14), or from naive Th cells by consecutive stimulations with IL-10 plus IFN-γ (15) or VitaminD3 plus corticosteroids (16).

Immature DC (iDC) reside as sentinel cells in mucosal tissues. Upon activation by microbial and/or inflammatory products, DC undergo a program of maturation, including the upregulation of the expression of peptide-loaded MHCII, costimulatory molecules (17) and signals that drive the development of either Th1, Th2 or unpolarized Th cell responses (18). The nature of these T cell polarizing signals is largely determined by the type of microbial and/or inflammatory products encountered in the peripheral tissues during their iDC phase. DC primed with viruses or intracellular bacteria often drive the development of protective Th1 cells, whereas helminth infections result in instruction for Th2 development (19)(Kapsenberg ML, NRI, in press).
DC are able to discriminate between different pathogenic compounds by the expression of various pattern-recognition-receptors (PRR) recognizing specific pathogen-associated-molecular-patterns (PAMP) (20). A well-studied example is the family of at least ten Toll-like receptors (TLR1-10), that are specifically stimulated by a number of structures present in either viruses, mycobacteria, gram negative or gram positive bacteria, mycoplasma's or parasites (21). An another example is the family of the C-type lectins (22), such as DC-SIGN and mannose receptor (MR), that recognize carbohydrate structures on pathogens and self-glycoproteins. Upon binding, the pathogens are internalized and processed for antigen presentation. In addition, signaling motifs in the cytoplasmatic tail of certain C-type lectins suggest that upon binding of the pathogen specialized signaling pathways can be switched on, influencing the effector function of DC (23)(van Kooyk Y, NRI, in press).

A rapidly growing number of reports suggest that certain pathogens can prime DC to develop into so-called regulatory DC, obtaining the capacity to drive the development of regulatory T cells. Regulatory T cells prevent autoimmunity and dampen excessive immune responses to pathogens, thereby preventing detrimental host tissue damage and allowing the generation of strong memory responses. In addition, certain pathogens may use enhanced induction of regulatory T cells as a strategy to evade immunity. For example, Plasmodium falciparum (24), Bordetella pertussis (25), Schistosoma mansoni (26), Mycobacteria tuberculosis (27) or Hepatitis C virus (28) modify DC function, and result in the generation of regulatory T cells in some of these cases.

In the present study we focused on the immunoregulatory capacity of lactobacilli. There are a few reports demonstrating in vitro modulation of DC or T cells responses, e.g. the induction of immunosuppressive cytokines IL-10 and TGFβ or suppressed T cell proliferation (29-31). In view of the above findings, we have investigated the putative capacity of probiotic lactobacilli to prime for regulatory DC.

Here we show that lactobacilli L. reuteri, L. casei and L. plantarum, bind to DC, but only L. reuteri and L. casei engage the C-type lectin DC-SIGN on DC. The binding of L.reuteri and L.casei to DC-SIGN is crucial for the ability of DC to prime for regulatory T cell development. The present results demonstrate that the therapeutical potential of selected probiotic bacteria may be based on their ability to induce tolerance by priming for regulatory DC via the ligation of DC-SIGN, supporting the development of regulatory T cells.
Material & Methods

Antibodies, cytokines and reagents

Human rIL-4 (sp. act. 1x10^8 U/mg) was obtained from PBH (Hanover, Germany). Human rGM-CSF (sp. act. 1.11x10^7 U/mg) was a gift of Schering-Plough (Uden, The Netherlands). The generation of DC-SIGN antibodies (AZN-D1 and (AZN-D2) was described before (32). HEK CD14, CD14/TLR2 and CD14/TLR4 cells were kindly provided by E. Kurt-Jones (University of Massachusetts Medical School, Worcester, MA). FLAG-tagged human TLR1 and TLR2 were provided by Tularic (San Francisco, CA). Non-tagged human TLR4 (hTOLL) in pcDNA3 was a gift from C. Janeway and R. Medzhitov (Yale University, New Heaven, CT) and was co-transfected with human MD-2 (0,25 μg DNA/transfection of TLR4 and MD-2), a gift from K. Miyake (University of Tokyo, Japan), FLAG-tagged TLR7 in pFLAG-CMV1 was a gift from D. Golenbock (University of Massachusetts Medical School) and FLAG-tagged TLR9 was a gift from S. Akira (Osaka University, Japan). Plastics were purchased from Greiner Bio-one (Alphen aan de Rijn, The Netherlands).

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, UK) containing 6,25% sheep blood. Lactobacilli were incubated at 37°C in a 5% CO2 atmosphere, and *Escherichia coli* at rT under aerobic conditions. After 3 days the bacterial growth of 2 densely seeded agar plates of each strain was harvested using a cotton swab, and suspended in 10 ml pyrogen-free PBS (Gibco Life Technologies, Breda, The Netherlands). After centrifugation at 4,000 x g for 10 min at rT, the pelleted bacteria were resuspended in 10 ml PBS, centrifuged and resuspended in 10 ml of PBS. One ml of this suspension was used to measure the optical density at 620 nm (OD620). An OD620 of 0,35 corresponded to 1 x 10^8 cfu for all test strains. The remaining suspension was centrifuged and the pelleted bacteria were resuspended to a concentration of 10^9 colony forming units (cfu) per ml in PBS. The suspensions were diluted to appropriate concentrations in PBS prior to further application.

In vitro generation and maturation of moDC

Immature DC were generated from monocytes (0,5x10^6 cells/well) in 24-well culture plates (Costar, Cambridge, MA) in Iscove's modified Dulbecco's medium (IMDM; Life Technologies Ltd., Paisley, UK) containing gentamycin (86 μg/ml; Duchefa, Haarlem, The Netherlands) and 10% FCS (Hyclone, Logan, UT), supplemented with rGM-CSF (500 U/ml) and rIL-4 (250 U/ml), as previously described (33). On day 6, maturation of iDC was induced by LPS (*E.coli*, Sigma-Aldrich, St. Louis, MO), different concentrations of lactobacilli, *E. coli* or Cordycepin (Sigma), in the presence or absence of the maturation factors (MF) IL-1β (25
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ng/ml), TNFα (50 ng/ml) (both purchased from Peprotech, Rocky Hill, NJ). After 48h, full maturation into CD83+ mature effector DC (mDC) was confirmed by flowcytometric analysis.

**Analysis of cell surface molecule expression by flow cytometry**

To analyze the phenotype of the acquired DC, the cells were incubated with PE-conjugated anti-CD86 (BD Pharmingen, San Diego, CA), PerCP-conjugated anti-HLA-DR (BD Biosciences, San Jose, CA) and APC-conjugated anti-CD83 (Caltag Laboratories, Burlingame, CA). Stained cells were analyzed by flow cytometry.

**Cytokine production by moDC**

Mature DC (2x10⁴ cells) were stimulated with mouse CD40L-expressing mouse plasmacytoma cells (J558 cells, 2x10³ cells; a gift from Dr. P. Lane, University of Birmingham, Birmingham, UK) in 200 µl culture medium. Supernatants were harvested after 24 h and stored at -20°C until cytokine levels were measured by ELISA. Determination of IL-12p70 concentrations in culture supernatants was performed by solid-phase sandwich ELISA, as previously described (34). Pairs of specific monoclonal antibodies and recombinant standards were obtained from BioSource International (Camarillo, CA) for the determination of IL-6, and from BD Pharmingen for IL-10 determination. The detection limits are as followed: IL-6, 20 pg/ml, IL-10, 20 pg/ml and IL-12p70, 3 pg/ml.

**Isolation of naive Th cells**

CD45RA+CD45RO- naive CD4+ T cells were isolated from PBMC through negative selection using CD4+ MACS MultiSort beads (Miltenyi Biotic, Bergisch Gladbach, Germany), supplemented with PE-labeled CD45RO-Abs (Dakopatts, Glostrup, Denmark). For subsequent depletion, the anti-hapten beads of the CD4+ isolation kit were supplemented with anti-PE coupled magnetic beads.

**Stimulation and culture of naive Th cells by effector DC**

Purified naive Th cells (2x10⁵ cells) were cocultured with mature DC (5x10³ cells) in 200 µl culture medium in the presence of the superantigen *Staphylococcus aureus* enterotoxin B (SEB) (10 pg/ml; Sigma), in 96-well flat-bottom culture plates (Costar). At day 5, rIL-2 (10 U/ml, Cetus Corp.) and rIL-15 (10 ng/ml, R&D Systems) were added and the cultures were expanded for the next 7 days.

**Cytokine production by Th cells**

On day 12, resting T cells were restimulated with PMA (10 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) for 6 h, the last 5 hrs in the presence of Brefeldin A (10 µg/ml; Sigma), to determine single-cell IL-4 and IFN-γ production by intracellular flowcytometric analysis. Cells were fixed in 2% paraformaldehyde (PFA; Merck, Darmstadt, Germany),
permeabilized with 0.5% saponin (ICN Biochemicals; Cleveland, OH) and stained with anti-human IFN-γ-FITC and anti-human IL-4-PE (both from BD Pharmingen).

**T cell suppressor activity**

On day 12, resting T cells were harvested and washed three times with serum-free medium. Cells (1x10^6) were stained with 3x10^5 M PKH-26 (Sigma), a red cell cycle tracking dye, for 5 minutes at room temperature according to the manufacturer's instructions. After thorough washing, 2.5x10^4 CD4^+ T cells (DC-primed T cells) were stimulated by anti-CD3 (1:5000 dilution of acite fluid) and anti-CD28 (0.5 μg/ml) in round-bottom 96-well plates. After overnight pre-activation, 2.5x10^4 peripheral CD4^+ T cells were added, representing the responder T cells. Prior to this, the responder T cells were labeled with CFSE (0.5 μM, Molecular Probes Inc., Eugene, OR), a green cell cycle tracking dye, for 15 min at room temperature. After 5 days, the content of PKH and CFSE in the DC-primed and responder T cells, respectively, was analyzed by flow cytometry.

**T cell proliferation**

Naive Th cells (5x10^4) were stimulated by LPS-, cordycepin or bacteria-treated mDC (5x10^3). Subsequently, cell proliferation was assessed by the incorporation of[^H]-TdR (Radiochemical Center, Amersham, Little Chalfont, U.K.) after a pulse with 13 KBq/well during the last 16 h of 5-day culture after stimulation, as measured by liquid scintillation spectroscopy.

**Stimulation of HEK cells transiently transfected with TLR constructs**

HEK-293 cells were transfected at 0.2x10^6 cells/well in 96-well plates (Nunc, Denmark) in DMEM culture medium (Biowhittaker, Verviers, Belgium) supplemented with 10% FCS and ciprofloxacin (10 μg/ml; Bayer) using PolyFect transfection reagent (Qiagen) with TLR-expressing plasmid (0.05 μg/well) and pELAM-luc (0.05 μg/well), a reporter construct that transcribes firefly luciferase from a NF-κB-dependent promoter, as previously described (Chow JC, JBC, 1999). After 24 hour, cells were stimulated with TNFα (100 ng/ml; Strathman Biotech, Hamburg, Germany), LPS (100 ng/ml, Sigma), PAM-3-CSK (10 μg/ml, EMC microcollections, Tuebingen, Germany), R848 (10 ng/ml, Invivogen, San Diego, CA), CpG-oligonucleotide (ODN) 2006 (10 μM, Biosource International Nivelles, Belgium), poly I:C (20 μg/ml, Sigma), or bacteria (10^7/ml). After 6 hours, cells were lysed in reporter lysis buffer (Promega, Madison, WI) and luciferase activity of the cellular lysate was measured using an assay kit from Promega according to the manufacturer's descriptions.

**Stimulation of cell lines Stably transfected with CD14/TLR2 and CD14/TLR4**
HEK-293 CD14 and HEK-293 CD14/TLR-9 cell lines were maintained in DMEM culture medium supplemented with 10% FCS, ciprofloxacin (10 μg/ml) and puromycin (5 μg/ml), while for the HEK-293 YFP-TLR-2 and HEK-293 pcDNA3 (TLR4) cell lines G418 (0,5 mg/ml) was used as selection marker. For stimulation experiments, cells were seeded at 0,2x10⁶ cells/well in 96 well flatbottom plates, and were stimulated after 24h with TNFα (100 ng/ml, Strahtman Biotech), LPS (100 ng/ml, Sigma) combined with 10 times diluted supernatant of MD-2 transfected cells, PAM-3-CSK (20 μg/ml), CpG-ODN 2006 (10 μM, Biosource International Nivelies) or bacteria (10⁷/ml). IL-8 production was measured in supernatants after 20h using a commercial kit (CLB, Amsterdam, The Netherlands) following the manufacturer’s instructions.

**DC or K562-cell binding by bacteria**

Bacteria (10⁹/ml) were labeled by incubation with FITC (0,5 mg/ml) in phosphate-buffered saline for 1h at room temperature. The FITC-labeled bacteria were washed three times to remove unbound FITC. Immature DC, K-562 cells or K-562-DC-SIGN cells (5x10⁶) were preincubated with neutralizing Abs to DC-SIGN (AZN-D1 and AZN-D2), mannosylated BSA (50 μg/ml, Sigma), Mannan (50 μg/ml, Sigma) or EDTA (10 mM) in TSM (20 mM Tris, 150 mM NaCl, 1mM CaCl₂, 2 mM MgCl₂, pH 8.0) + 0,5% BSA for 10 min at room temperature. Thereafter, the FITC-labeled bacteria (ratio bacteria: cells of 10:1) were added and incubated for 45 min at 37°C. After washing, the cells were analyzed by flow cytometry.

Immature DC were generated by culturing monocytes in RPMI-1640/10% FCS in the presence of IL-4 (500 U/ml, Schering-Plough, Kenilworth, NJ) and GM-CSF (800 U/ml, Schering-Plough) for 5-8 days (32). K-562 cells and K-562-DC-SIGN cells were cultured as described (32).

**Statistics**

Data are expressed as mean ± SEM. Data were analyzed for statistical significance with GraphPad InStat® software (version 3.00; GraphPad InStat, Inc., San Diego, CA). using ANOVA followed by Bonferroni’s multiple comparison test or a non-parametric unpaired Student t test to compare two sets of data or variables between two groups, respectively. A P value < 0,05 was considered as the level of significant.

**Results**

**Lactobacilli induce partial DC maturation**

The three different lactobacillus species, *L. reuteri, L. casei* and *L. plantarum* were randomly selected for a comparative study regarding their immunoregulatory effect on DC function, using *E. coli*, a gram-negative gutflora commensal, for comparison. Different concentrations of E.coli were tested to determine the optimal concentration required for full maturation, based on the increase of the co-stimulatory molecule CD86, MHCII molecule HLA-DR and maturation marker CD83. Full maturation was obtained with 10⁷ bacteria (ratio
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100:1), whereas 10^6 bacteria (ratio 10:1) gave only partial maturation and 10^5 bacteria (ratio 1:1) did not induce maturation (data not shown). Compared to 10^7 E. coli bacteria, 10^7 lactobacillus bacteria had only a partial effect on the expression of these molecules (figure 1A, left panel). To determine the capacity of the different bacteria to block DC maturation, different numbers of bacteria were added to DC during their maturation induced by the cytokines IL-1β and TNFα (maturation factors or MF). Even at the highest concentration (10^7 bacteria), the different lactobacilli did not inhibit MF-induced maturation (figure 1A, right panel). Moreover, the CD40L-induced production of IL-12, IL-10 or IL-6 in MF-treated DC was not significantly affected by the lactobacilli (figure 1B). In contrast, at this bacterial load, E. coli induced strong upregulation of IL-10 and IL-6 production as described before (H.H. Smits, manuscript submitted).

**Figure 1: Lactobacilli induce partial DC maturation.**
Immature DC were generated as described elsewhere (33). (A) Maturation was induced by addition of LPS (100 ng/ml), 10^7 cfu bacteria, LPS plus MF (rIL-1β (25 ng/ml) and rTNFα (50 ng/ml)), or MF plus 10^7 cfu bacteria. After 48 h, mDC were harvested, washed and (A) surface expression of CD86, HLA-DR and CD83 was analyzed by flowcytometry. (B) Mature DC (2.10^6 cells/well) were stimulated with mouse CD40L-expressing mouse plasmacytoid cells (J558 cells, 2x10^5 cells/well) to induce the production of IL-12, IL-10 and IL-6. After 24h, supernatants were collected and IL-12p70, IL-10 and IL-6 production were measured by ELISA.
**L. reuteri** and **L. casei** induce regulatory T cell development by modulation of DC function

Next, the immunoregulatory potential of the different lactobacilli on the Th cell polarizing capacity of DC was investigated. To this end, naïve T cells were stimulated with superantigen SEB plus DC matured by MF (mDC) in the presence or absence of lactobacilli or E. coli. After 12 days, the intracellular cytokine profile of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 was determined in the developed effector Th cells. Clearly, the cytokine profile in the T cells stimulated by lactobacilli-primed DC was similar to the cytokine profile of effector Th cells stimulated by the control DC matured by MF plus LPS (Figure 2A). The inability of Lactobacilli to bias the Th1/Th2 polarizing capacity of DC is in line with previous findings with other gram-positive gut flora bacteria (H.H. Smits et al, manuscript submitted). In comparison, as previously described (H.H. Smits et al, manuscript submitted), *E. coli* primed for enhanced Th1 development. Although the cytokine profile of the effector T cells generated by lactobacilli-primed mDC was unchanged in comparison to that of the control T cells, the T cells responding to the lactobacilli-primed mDC proliferated less vigorously in comparison to the T cells primed with MF/*E.coli*- or MF/LPS-matured DC (figure 2B).

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 a red cell cycle tracking dye, PKH-26, and stimulated with anti-CD3 and anti-CD28 and cocultured with peripheral CD4+ T cells (responder T cells) labeled with CFSE, a green cell cycle tracking dye, in the presence of anti-CD3 and anti-CD28. Suppression is evident when responder T cells proliferate slower and, consequently, lose less CSFE on the single cell basis than positive controls. The reliability of this assay was supported in a previous study with effector T cells generated by coculture with mDC matured with or without the suppressive compound cordycepin (negative control; H.H. Smits and E.C. de Jong et al, manuscript in preparation). After 5 days, flowcytometric analysis showed that the levels of CFSE in responder T cells were much higher after their co-culture with effector T cells generated with cordycepin-primed DC, indicating a lower proliferation. Thus, cordycepin-primed DC induce regulatory T cells that inhibit proliferation of responder T cells (figure 2C).

CSFE levels were substantially higher in responder cells cocultured with effector T cells generated in response to *L. reuteri*- or *L. casei*-treated mDC in comparison to responder cells cocultured with effector T cells generated in coculture with MF plus LPS-, *L. plantarum*- or *E. coli*-treated mDC (figure 2C). The mean fluorescence intensity (MFI) of responder T cells cocultured with effector T cells primed by MF/LPS-DC was set at 100%, representing the maximal proliferation. This value was used to calculate the relative proliferation for each condition by comparing it to the MFI of the responder T cells cocultured with other DC-primed T cells (figure 2D). Different doses of bacteria-treatment were evaluated: $10^3 - 10^5$ –
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$10^7$ cfu bacteria, showing a maximal inhibitory effect for $10^5$ cfu *L. reuteri* and *L. casei* bacteria. Neither *E. coli* nor *L. plantarum*-DC-primed T cells inhibited responder T cell proliferation, independent of the bacterial dose (figure 2D). In summary, these data indicate that *L. reuteri* and *L. casei* modulate DC function and prime for regulatory T cell development, which appears to be optimal under conditions of low doses of bacteria (ratio bacteria: DC of 1:1).

**2A**

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**2B**

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**2C**

MF/ LPS  
MF/ E. coli  
MF/ Cordycepin  
MF/ L. reuteri  
MF/ L. casei  
MF/ L. plantarum  
CFSE
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Figure 2: L. reuteri and L. casei induce regulatory T cell development by modulation of DC function. Generation of iDC and maturation conditions are described in the legend to figure 1. (A) Mature DC (5x10^5 cell/well) were cocultured with naive Th cells (2x10^5 cells/well) and superantigen SEB (10 pg/ml). After 12 days, IFN-γ and IL-4 productions per cell were analyzed by intracellular FACS-staining following a 6h PMA/ionomycin stimulation, the last 5h in the presence of Brefeldin A. (B) Mature DC (2,5x10^5 cell/well) were cocultured with naive Th cells (5x10^4 cells/well). Cell proliferation was assessed by the incorporation of [3H]-Tdr after a pulse with 13 KBq/well during the last 6 day of culture after stimulation, as measured by liquid scintillation spectroscopy. (C) Naive Th cells were stimulated as described in part A. After 12 days, the DC-primed T cells were labeled with 3x10^5 M PKH-26, and stimulated with suboptimal concentrations of anti-CD3 (1:50000 acetes-fluid) and anti-CD28 (0,5 μg/ml). After overnight incubation, CFSE-labeled responder T cells (peripheral CD4^+ T cells), were added in a 1:1 ratio (2,5x10^4 each). After 5 days the PKH-26 and CFSE staining of the cells were analyzed by flowcytometry. The gray shade CFSE profile represents the test condition (bacteria: 10^5), whereas the overlay indicates the proliferation in the presence of control DC-primed T cells (MF/LPS). The figure is a representative out of 7 independent experiments. (D) The MFI of the CFSE-labeled responder cells cocultured in the presence of control test cells (MF/LPS) was set at 100%, representing the maximal proliferation. The MFI of the responder T cells cocultured with other DC-primed T cells was compared to this value, calculating the relative proliferation. The results are expressed as the mean percentages ± SEM from 4-7 independent experiments (Cordycepin: 25 μg/ml). Data were analyzed for statistical significance using ANOVA followed by Bonferroni's multiple comparison test. * P<0,05, ** P<0,01.

TLR activation by lactobacilli

Lactobacilli, the gram positive control bacterium Staphylococcus aureus, and gram negative E. coli were evaluated for their TLR-activating potential in stably transfected HEK-293 cells (CD14, CD14/TLR2 and CD14/TLR4) and transiently transfected HEK-293 cells (TLR1, TLR2, TLR4 + MD-2, TLR6, TLR7and TLR9) by determining IL-8 production in these cells or luciferase activity, respectively. In transient transfection experiments, several positive controls were analyzed, PAM3CSK for TLR2 (fold increase: 90), LPS for TLR4 (fold increase: 47), R848 for TLR7 (fold increase: 4,5) and CpG for TLR9 (fold increase: 5) (data not shown). E. coli ligated TLR2 and TLR4, and TLR1, TLR6 and TLR9 to a lesser extent,
whereas \textit{S. aureus} only ligated TLR2 (figure 3A). These data were confirmed in the stable transfectants (figure 3B). In contrast, the three lactobacilli did not activate any of the TLR tested, except for some TLR4 activation by \textit{L. casei}, only detected at a high dose of bacteria \((10^7 \text{ cfu}; \text{lower doses not show})\). These results suggest that the Lactobacilli tested here do not activate DC via ligation of TLR (figure 3A and figure 3B).

\textbf{L. reuteri and L. casei ligate DC-SIGN on moDC}

Next, we evaluated which other PRR could interact with these lactobacilli. C-type lectins are highly expressed by DC and bind carbohydrate structures on pathogens in a cation dependent manner (van Kooyk Y, NRI, in press). Whereas \textit{E. coli} did not bind iDC, the three lactobacilli bound to iDC at different degrees. This binding was strongly inhibited by EDTA implying a role for C-type lectins in calcium-dependent carbohydrate recognition (figure 4A). In search for DC-associated molecules that may 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 or K-562 cells, stably transfected with

\textbf{Figure 3: TLR activity by Lactobacilli.} (A) HEK 293 cells were transiently transfected with human TLR1, TLR2, TLR3, TLR4 + MD2, TLR6, TLR7 and TLR9, together with an ELAM-luciferase reporter construct and were stimulated with \(10^7\) bacteria or TNF\(\alpha\) (100 ng/ml). Luciferase activity was determined 6h after stimulation. (B) Stable transfected HEK 293 cells with either TLR2 or TLR4 were seeded at \(0,2 \times 10^6\) cells/well and stimulated with \(10^7\) bacteria/ml or TNF\(\alpha\) (100 ng/ml). After 24 hours, supernatant was taken and IL-8 content was analyzed by ELISA.
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DC-SIGN. After substraction of Mock-binding, L. casei and L. reuteri, both inducing the development of regulatory T cells, but not L. plantarum, bound in variable amounts (L. casei high and L. reuteri low) to DC-SIGN-expressing cells (figure 4B). Blocking studies demonstrated that L. reuteri and L. casei specifically interact with DC-SIGN as neutralizing anti-DC-SIGN or EDTA blocked the binding completely (figure 4B). 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 iDC, we performed binding assays of lactobacilli to iDC 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 or 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, as incubation with mannosylated BSA (ligates selectively to MR) did only weakly affect the binding of the L. casei to iDC, in contrast to the other bacteria in which the DC binding was not affected at all (figure 4C). In summary, these findings demonstrate that all three lactobacilli bind iDC possibly through C-type lectin receptors. Interestingly, only the bacteria (L. reuteri and L. casei) that selectively prime DC for the capacity to drive the development of regulatory T cells, do specifically ligate DC-SIGN.
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**Figure 4: L. reuteri and L. casei ligate DC-SIGN on moDC.** (A) Immature DC (5x10^4) were incubated with FITC-labeled bacteria (5x10^5), after a prior 10 min preincubation with TSM + 0.5% BSA or EDTA (10 mM), for 45 min at 37 °C. After washing, the cells were analyzed by flow cytometry. (B) Similar protocol, as described in the part A, was used to analyze bacteria binding to DC-SIGN-transfected K562 or untransfected K562 cells. Preincubations were performed with either TSM + 0.5% BSA, EDTA (10 mM) or anti-DC-SIGN (AZN-D1 and AZN-D2, 20 µg/ml). The results are expressed as the difference in binding to the transfected and untransfected K562 cells and represent the mean ± SEM out of 3 independent experiments. (C) DC-binding was performed as described in part A. Preincubations were performed with either TSM + 0.5% BSA, anti-DC-SIGN (AZN-D1 and AZN-D2, 20 µg/ml), mannosylated-BSA (50 µg/ml), Mannan (50 µM). The results are expressed as the mean percentages ± SEM of 4 different donors. The binding of the individual bacteria was set at 100% and the relative binding of bacteria in other test conditions was compared to this value.
DC-SIGN ligation is crucial for modulation of DC function by L. reuteri and L. casei

Next, we investigated whether pathogen targeting to DC-SIGN contributes to priming for regulatory T cell development by adding blocking antibodies to DC-SIGN or mannan during the maturation of the DC by MF plus L. reuteri and L. casei. Subsequently, cocultures of bacteria-primed mDC and naive Th cells were performed and the resulting effector T cells analyzed for their capacity to inhibit responder T cell proliferation. The blocking of ligation of L. reuteri and L. casei to DC-SIGN, with either neutralizing antibodies or mannan, abolished the priming for regulatory T cell development, since the proliferation of the responder cells was almost completely restored. (figure 5). Proliferation of responder T cells cocultured with effector T cells generated in the presence of L. plantarum or E. coli-primed DC was not affected by blocking antibodies to DC-SIGN or mannan. These results demonstrate that DC-SIGN ligation by L. reuteri and L. casei is crucial for DC to acquire their regulatory function.

![Figure 5](image)

**Figure 5:** Blocking of DC-SIGN interaction with L. reuteri and L. casei abrogates the priming for regulatory T cell development. Generation of iDC is described in the legend to figure 1. Immature DC were preincubated (30 min, 37 °C) with either isotype control Ab (20 μg/ml), anti-DC-SIGN (AZN-D1; 20 μg/ml) or Mannan (50 μM). Thereafter maturation was conducted as described in the legend to figure 1. Induction of suppressor cell activity was analyzed as described in the legend to figure 2.

**Discussion**

The present study on three probiotic lactobacilli demonstrates that L. reuteri and L. casei, in contrast to L. plantarum, prime DC to promote the development of regulatory T cells. Experiments with TLR-transfectants proved that all three Lactobacilli tested did not activate TLR, except for L. casei that induced some TLR4 activity. However, L. reuteri and L.
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casei both potently inducing the development of regulatory T cells, were captured by DC-SIGN on DC, an interaction that appeared to be crucial for the priming of regulatory DC.

We here demonstrate for the first time that ligation of DC-SIGN can mediate the induction of regulatory T cells. So far, various microorganisms have been described to bind DC-SIGN, including mycobacteria and its constituent ManLAM (32,35). Ligation of DC-SIGN by mycobacteria or ManLAM can modulate DC function as well (36). ManLAM inhibits LPS-induced DC maturation and induces IL-10 production. In sharp contrast, L. reuteri and L. casei, even at the high dose of $10^7$ cfu bacteria, do not inhibit DC maturation and hardly affect the production of the cytokines IL-12, IL-10 or IL-6 production. Strikingly, the regulatory T cell polarizing capacity of lactobacillus-primed iDC (DC primed by bacteria in the absence of MF) was clearly less in comparison to lactobacillus-treated mature DC (mDC; DC primed by bacteria in the presence of MF) (data not shown). These findings suggest that, in addition to regulatory signals via DC-SIGN, also activating signals are necessary to induce regulatory DC. Apparently, DC-SIGN cannot induce regulatory DC independently, but acts via cross-regulation of positive signaling pathways (37). At present, it is unknown which signals are transduced by DC-SIGN ligation.

Another consequence of the finding that lactobacillus-primed mDC are far more efficient than lactobacillus-primed iDC in the induction of regulatory T cell development, is that probiotic-primed mDC express molecules that are crucial for an optimal capacity to drive regulatory T cell development. At present, the identity of these molecules is unknown and is currently under investigation. Neutralization experiments with blocking IL-10 Abs did not support the involvement of DC-derived IL-10 in this respect (data not shown).

DC-SIGN has a high affinity for mannose and fucose residues, which are differentially expressed in glycosylated surfaces of different pathogens (35). At present, the composition and identity of carbohydrate structures on the different lactobacilli is unknown, as well as how this is related to other DC-SIGN ligands. However, it can be speculated that the functional DC-SIGN-related differences seen by the various micro-organisms, can be explained by different combinations and/or affinities of mannose and/or fucose residues binding to DC-SIGN or additional lectins.

Probiotic bacteria will encounter DC in the mucosal areas of the intestines. These DC express high levels of DC-SIGN (32,38). Although the lactobacilli tested here do not induce DC maturation themselves, apparently these DC become mature in the mucosal environment, as DC in the draining lymph nodes of the intestines, i.e. the iliac and mesenteric lymph nodes clearly have a mature phenotype (39). This finding is easily explained, as the gutflora consists of many different bacteria, including species (i.e. E. coli) that do activate PRR (i.e. TLR) that confer DC maturation. In addition, tissue cells in the microenvironment of DC produce pro-inflammatory cytokines in response to those bacteria, creating an environment in which cytokines such as IL-1β and TNFα (MF) will be present. All
these factors combined will create the ideal setting for probiotic bacteria to prime maturing DC for regulatory T cell development via interaction with DC-SIGN.

Previous studies have demonstrated that, in addition to microbial priming, the characteristics of the tissue, i.e. in the gut, can affect the T cell polarizing capacity of DC as well. For example, DC isolated from Peyer’s patches do not produce IL-12, but produce increased amounts of IL-10, and appear to drive Th2 responses (40). Furthermore, gut DC isolated from mesenteric lymph nodes of OVA-fed mice induce regulatory T cells via the secretion of TGFβ (41). These studies indicate that in particular conditions mucosal DC can acquire the capacity to drive regulatory T cell development. This type of response reflects a plausible way of preventing excessive immune responses to innocuous proteins and non-pathogenic microorganisms to which mucosal surfaces are constantly exposed. Although we did not have the opportunity to test the plasticity of gut DC in our models, it is tempting to speculate that in vivo exposure to probiotic bacteria may more readily prime gut DC for regulatory T cell development and help to prevent local excessive immune pathology.

Our study clearly demonstrated that optimal priming for regulatory DC occurs at $10^5$ cfu bacteria, which is at a bacteria:DC ratio of 1:1, and that a significantly lower priming effect occurs at bacteria:DC ratio of 100:1 or 10:1 (data not shown). This is an intriguing finding, suggesting that at high doses these bacteria may activate additional pathways in DC. These pathways may interfere with the cross talk of DC-SIGN signaling, that in the end may (partially) prevent regulatory T cell development. It can be speculated that this/these pathway(s), triggered at high bacterial (even probiotic bacterial) doses, represent(s) an escape to clearance of bacterial overload, overruling the generalized immune suppression prevailing at low bacterial load.

Probiotic bacteria are interesting candidates in therapy of certain inflammatory diseases, such as gastroenteritis and allergic diseases, in particular atopic dermatitis (1-3). A first series of clinical studies with various probiotic bacteria has shown promising results with respect to decreases in inflammation and disease symptoms. It is attractive to suggest that for some bacteria these beneficial effects may, at least in part, be explained by the priming of DC for regulatory T cell development. However, caution must be taken, as these in vivo effects may 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 regulatory T cells is crucial in the amelioration of disease, these therapies may be improved by the prior screening of probiotic bacteria for their potential to induce regulatory T cells. Furthermore, if more detailed knowledge of the mechanisms underlying their effect is available, it may well be possible to improve therapy by using cocktails of different probiotics, combining specialized regulatory features of the various probiotics.
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