

Direct regulatory immune activity of lactic acid bacteria on Der p 1-pulsed dendritic cells from allergic patients

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Background: Lactic acid bacteria (LAB) are suggested to play a regulatory role in the development of allergic reactions. However, their potential effects on dendritic cells (DCs) directing the immune polarization remain unclear.

Objective: The immunologic effect of *Lactobacillus plantarum* NCIMB 8826 (LAB1) on monocyte-derived dendritic cells (MD-DCs) from patients allergic to house dust mite was evaluated.

Methods: MD-DCs were stimulated for 24 hours with the related allergen Der p 1 in the presence or absence of LAB1. Cell-surface markers were assessed by means of FACS analysis, and the key polarizing cytokines IL-12 and IL-10 were quantified. The subsequent regulatory effect of pulsed MD-DCs on naive or memory T cells was evaluated by determining the T-cell cytokine profile.

Results: LAB1 induced the maturation of MD-DCs, even if pulsed with Der p 1. Interestingly, after incubation with LAB1 and Der p 1, MD-DCs produced higher amounts of IL-12 than Der p 1-pulsed DCs. Indeed, the T_H2 cytokine (IL-4 and IL-5) production observed when naive or memory autologous T cells were cocultured with Der p 1-pulsed MD-DCs was highly reduced in the presence of LAB1. Finally, in contrast to naive or memory T cells exposed once to Der p 1-pulsed DCs, T cells stimulated by MD-DCs pulsed with Der p 1 and LAB1 failed to produce T_H2 cytokines in response to a new stimulation with Der p 1-pulsed DCs.

Conclusion: Thus in the presence of LAB1, MD-DCs from allergic patients tend to reorientate the T-cell response toward a beneficial T_H1 profile. (J Allergy Clin Immunol 2005;116:198-204.)

Key words: Dendritic cells, T_H1/T_H2 cells, bacteria, allergy

Abbreviations used

DC: Dendritic cell

LAB: Lactic acid bacteria

LAB1: *Lactobacillus plantarum* NCIMB 8826

MD-DC: Monocyte-derived dendritic cell

PFA: Paraformaldehyde

TLR: Toll-like receptor

Dendritic cells (DCs) are professional antigen-presenting cells, and because of their capacity to stimulate T cells, they have a key role in the initiation and regulation of immune response. Various *in vitro* and *in vivo* studies demonstrated their pivotal involvement in the development of allergic responses to relevant allergens^{1,2} characterized by a predominant T_H2 profile (potential IL-4, IL-5, IL-9, and IL-13 production).³ Several recent reports emphasized the increase of allergic diseases in the industrialized countries. Among the different hypotheses, modification in gut microflora bacteria composition was suggested as an explanation of the immune tolerance disruption⁴ required for the development of allergy. Thus children in Estonia, where the prevalence of allergy is much lower than in Sweden, have higher amounts of lactic acid bacteria (LAB) in their bowel flora. Moreover, it has been proposed that DCs localized in the gut-associated lymphoid tissue can sample bacteria from the intestinal lumen and modify the immune homeostasis.⁵

Lactobacilli, which are gram-positive, nonpathogenic commensal organisms, constitute an important part of the intestinal microflora and antagonize pathogens. Apart from their protective effect against several infectious diseases, some LAB strains were reported to reduce some allergic manifestations. Thus administration of *Lactobacillus rhamnosus* GG to breast-feeding mothers and babies led to a risk reduction of 50% in the development of atopic eczema.⁶ We have demonstrated that the T_H2 cytokine production by PBMCs from patients with house dust mite allergy stimulated with related allergen was highly reduced in the presence of LAB strains. This antiallergic effect was dependent on antigen-presenting cells and required IL-12, a key cytokine in the orientation of the immune response toward a T_H1 profile.⁷

In the present study we have mainly evaluated the potential immune modifications that LAB might directly

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induce at the level of DCs from allergic patients. Indeed, the development of a defined T-cell profile is highly dependent on the DC-stimulating environment. Thus we have compared the reactivity of monocyte-derived dendritic cells (MD-DCs) from patients allergic to house dust mite either pulsed with Der p 1 or costimulated with Der p 1 and *Lactobacillus plantarum* NCIMB 8826 (LAB1). Our data showed that the presence of LAB1 during the allergen pulse allowed allergic MD-DCs to produce IL-12 and to exhibit an anti- T_H2 effect. Whereas the phenomenon was observed on both naive T cells and memory T cells, LAB1 tended to generate T cells refractory to a further allergen stimulation. This direct effect of LAB1 on allergic MD-DCs underlines the potential and natural regulatory activity of such LAB on both the initiation and the development of the allergic reaction.

METHODS

Patients

Blood was collected from allergic patients sensitive to *Dermatophagoides pteronyssinus* and from healthy donors. All allergic patients had a history of asthma and presented the usual features of house dust mite sensitization: specific IgE antibodies (RAST class >3) and positive skin prick test responses to *D pteronyssinus*. The level of total IgE was greater than 250 kU/L. Healthy donors did not display any of these characteristics: total IgE level of less than 20 kU/L and absence of specific IgE for *D pteronyssinus* in serum (level <0.35 kU/L).

Cell preparations

After depletion of platelet-rich plasma, blood was diluted in RPMI 1640 (1:1; Live Technologies, Paisley, Scotland) and deposited over a Ficoll density gradient (Pharmacia, Uppsala, Sweden). After centrifugation (400g for 30 minutes), human PBMCs were isolated, washed, and resuspended in PBS, pH 7.2, supplemented with 0.5% BSA (SIGMA, Saint-Quentin Fallavier, France) and 2 mM EDTA. PBMCs were incubated on ice for 30 minutes with anti-CD14 antibodies coupled to magnetic micro-beads (Miltenyi Biotec, Bergisch Gladbach, Germany), washed, and applied onto a column placed in a magnetic MACS separator (Miltenyi Biotec, Paris, France). After elution, CD14⁺ cells were resuspended in RPMI 1640 containing 10% heat-inactivated FCS (Bio Media, Canada), 2 mM L-glutamine, and antibiotics (Ticarpen 1%; Smith Kline Beecham, Belgium) before plating (3×10^6 cells/3 mL per well) into 6-well flat-bottomed culture plates. CD14⁺ cells were differentiated into MD-DCs for 6 days in medium supplemented with GM-CSF (20 ng/mL; Peprotech, United Kingdom) and IL-4 (200 IU/mL; R&D Systems, United Kingdom).

Naive CD45RA⁺CD4⁺ or memory CD45RO⁺CD4⁺ T cells were isolated from the eluted CD14⁺ cell fraction by using a CD4⁺ T-cell isolation kit (containing CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies, Miltenyi Biotec) supplemented with either CD45RO or CD45RA MACS microbeads. T cells are frozen in FCS containing 10% DMSO until used.

Preparation of bacteria

LAB1 was cultured overnight in sterile MRS broth medium (Difco, Detroit, Mich). The LAB1 suspension was diluted 1:20 in fresh sterile medium and further cultured until the exponential phase, which was verified by measuring OD at 600 nm. LAB1 was washed twice in LPS-free sterile PBS, resuspended in LPS-free sterile PBS

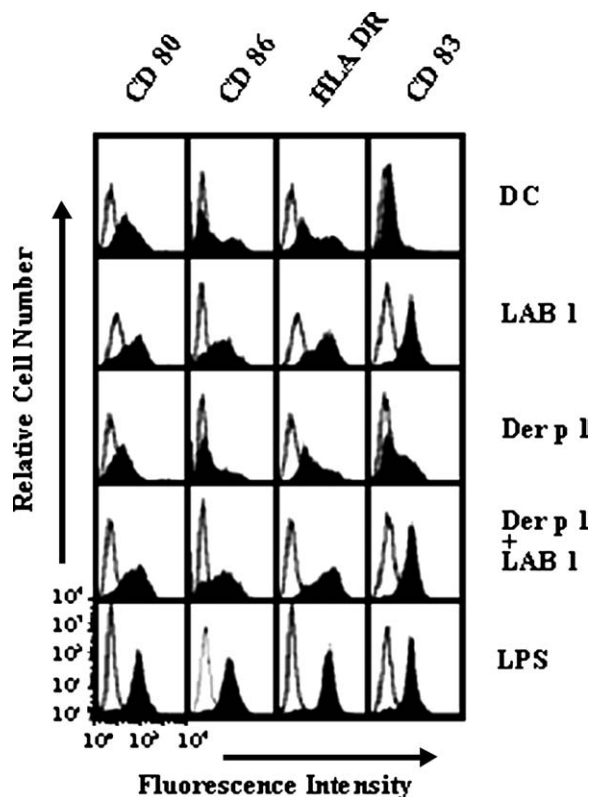


FIG 1. Phenotype of MD-DCs. MD-DCs were pulsed either with Der p 1 (1 μ g/mL), LAB1 (1:10), or both for 24 hours and analyzed by means of flow cytometry for CD80, CD86, CD83, and HLA-DR expression. Open histograms represent the cell reactivity to fluorochrome-matched isotype control antibodies. One representative experiment of 5 is shown.

supplemented with 20% glycerol, and stored at -80°C . The concentration of bacteria was determined by plating aliquots of serial dilution. LAB prepared in such a way demonstrated a similar effect on dendritic cells from a Toll-like receptor (TLR) 4-deficient mouse or a TLR4(+/+) mouse. Fixed bacteria were prepared by means of 15 minutes of incubation in LPS-free paraformaldehyde (PFA) 4% solution at room temperature and washed 3 times in LPS-free sterile PBS. Plating a sample on MRS medium containing agar was used to check the absence of viable bacteria. The potential presence of endotoxin in the LAB preparations was evaluated by using the quantitative chromogenic Limulus Amebocyte Lysate test (QCL-1000) in the presence or absence of β -glucan blocker to avoid false-positive results (BioWhittaker, Walkersville, Mass). No significant endotoxin level was detected in the LAB preparations (<0.1 EU/mL).

Activation of DCs

MD-DCs (1×10^6 cells/1 mL per well) were incubated for 24 hours either with Der p 1 (1 μ g/mL) or PFA-fixed LAB1 at 2 ratios (1 bacteria [1:1] or 10 bacteria [10:1] per DC) or with both stimuli concomitantly. As a positive maturation control, DCs were stimulated with LPS (1 μ g/mL; Sigma, Saint-Quentin Fallavier, France).

DC surface marker analysis

Cells were collected, washed twice in PBS, and incubated for 30 minutes at 4°C with mAbs: FITC-conjugated anti-CD86 and anti-CD1a, phycoerythrin-conjugated anti-CD80 and anti-HLA-DR

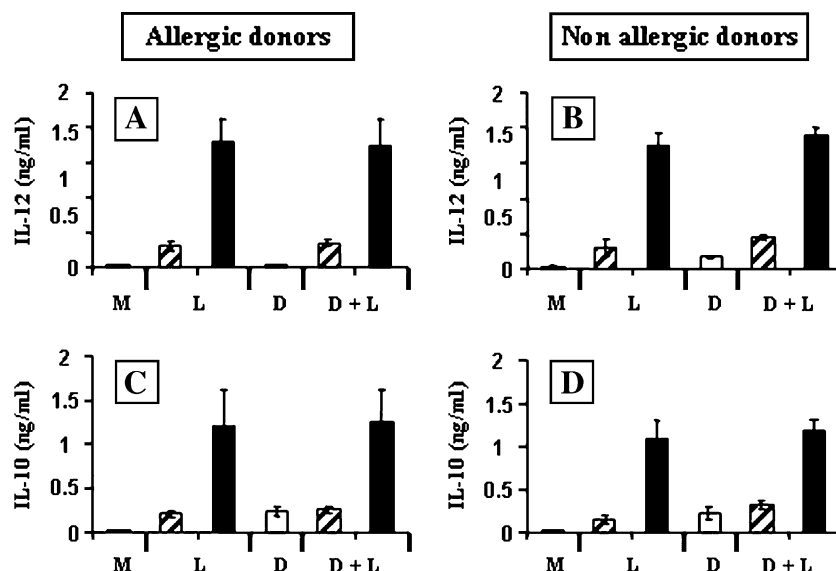


FIG 2. LAB1 effect on the IL-10 and IL-12 production by Der p 1-pulsed DCs. MD-DCs from allergic (A and C) or healthy (B and D) donors were incubated for 24 hours with Der p 1 (D), LAB1 (L), or both (D+L; bacteria/DC ratios, 1:1 [hatched columns] or 10:1 [filled columns]) or alone in medium (M). IL-10 and IL-12 concentrations determined by means of ELISA were presented as means $\pm$ SD values ($n = 6$). *Statistical differences ($P \leq .05$).

(Becton Dickinson, Mountain View, Calif), and phycoerythrin-conjugated anti-CD83 (Beckman Coulter, Immunotech, Marseille, France). Isotype controls were performed by using irrelevant mAbs. After washing, cells fixed in a PFA solution (1%) were analyzed with a FACS Calibur (Becton Dickinson).

Cytokine assay

DC activation. After a 24-hour stimulation in the 4 aforementioned conditions (unpulsed or pulsed with Der p 1, LAB, or Der p 1 plus LAB), supernatants from MD-DCs (1×10^6) were collected, centrifuged, and tested by means of ELISA for the presence of IL-10 or IL-12 (IL-12 p70; Diaclone, Besançon, France). The sensitivity of both assays was 5 pg/mL.

T cell-DC cocultures. After washing, 1×10^5 stimulated MD-DCs were incubated either with 1×10^6 CD45RO⁺ T cells or with 1×10^6 CD45RA⁺ T cells for 2 and 5 days, respectively. The cocultures were performed in 48-well flat-bottomed culture plates (1 mL/well). To evaluate the capacity of previously stimulated T cells to respond to a new allergen presentation, collected T cells were washed and further incubated for 24 hours with 10^5 fresh autologous Der p 1-pulsed DCs (ratio DC/T, 1:10). The presence of IL-4 (Diaclone), IL-5 (PharMingen), and IFN- γ (Diaclone) in supernatants was detected by means of ELISA (respective sensitivity of assays: 0.5, 5, and 5 pg/mL).

Proliferation assay

Freshly purified T cells were labeled with 5-carboxy-fluorescein diacetate succinimidyl ester (0.1 mM; Molecular Probes, Eugene, Ore) at 37°C for 10 minutes and washed twice in RPMI 1640 containing 10% FCS. At days 0 and 5 of T-cell-DC coculture, cells were labeled with Cychrome-conjugated anti-CD3 (Becton Dickinson) and washed twice, and T-cell proliferation was evaluated by means of flow cytometry.

Statistical analysis

Parametric statistical analysis was performed with the Student test. P values of .05 or less were considered statistically significant.

RESULTS

Der p 1-pulsed MD-DCs undergo maturation in the presence of LAB1

In the presence of the higher dose of LAB1, MD-DCs displayed a mature cell phenotype similar to that of LPS-stimulated DCs, which was characterized by an increase in CD80, CD86, CD83, and HLA-DR expression (Fig 1) and a decrease in the CD1a marker (data not shown). The lower dose of bacteria (ratio, 1 bacteria/1 DC) induced only a slight increase in the expression of all markers tested (data not shown). In our experimental conditions, when LAB1 was added to Der p 1-stimulated MD-DCs, the phenotype profile was similar to that detected with DCs stimulated with LAB1 alone.

Effect of LAB1 on IL-10 and IL-12 production by DCs stimulated with Der p 1 allergen

The DC maturation process is associated with the secretion of defined cytokines that direct the T-cell profile. IL-12 and IL-10 produced by MD-DCs pulsed only with Der p 1 or LAB1 or with both Der p 1 and LAB1 were evaluated. The reactivity of DCs to Der p 1 was different in allergic patients and healthy donors. Whereas in allergic patients Der p 1-pulsed DCs failed to secrete significant amounts of IL-12, DCs from healthy donors produced IL-12 (Fig 2). Interestingly, in allergic patients LAB1 stimulated IL-12 production by Der p 1-pulsed DCs, and the IL-12 levels remained similar to those obtained in the absence of Der p 1. Moreover, LAB1 induced a dose-dependent IL-10 production, which remains at similar level in the presence of Der p 1. It is noteworthy that in allergic patients and healthy donors, according to the IL-12 and IL-10 production level, the DC reactivity toward

LAB1 seemed to be quite equal. Thus LAB1 was demonstrated to induce DCs from allergic patients to produce the pro- T_H1 cytokine IL-12, as observed in healthy donors.

Effect of LAB1 on T_H2 cytokine production by CD4-naïve T cells

To evaluate the influence of LAB1 on T-cell polarization, Der p 1-pulsed MD-DCs incubated or not with bacteria were further cocultured with autologous CD4-naïve T cells. The production of T_H1 cytokines (IFN- γ) and T_H2 cytokines (IL-4 and IL-5) was analyzed. In allergic patients, when naïve T cells were incubated with Der p 1-pulsed DCs, production of IL-4 and IL-5 was observed, but this was not the case with unpulsed DCs (Fig 3). In contrast, IL-4 and IL-5 production remained undetectable in healthy donors (data not shown). In allergic patients stimulation of naïve T cells with DCs pulsed only with LAB1 induced a high and dose-dependent increase in IFN- γ production, and addition of Der p 1 to LAB1 stimulus did not significantly modify IFN- γ secretion. However, interestingly, in allergic patients LAB1 decreased the production of T_H2 cytokines induced by Der p 1. This inhibition was dose dependent and became significant only with the higher dose of LAB1. These results were correlated with an increase of the percentage of IFN- γ -secreting T cells and a decrease of IL-4-secreting T cells (data not shown). Thus in allergic patients LAB1 can inhibit the allergen-induced T_H2 cytokines produced by naïve T cells and might favor the orientation of the response toward a T_H1 phenotype.

Effect of LAB1 on T_H2 cytokine produced by CD4 memory T cells

We next evaluated the potential effect of LAB1 on a so-called ongoing immune response by measuring cytokines produced by DC-stimulated memory T cells. MD-DCs from allergic patients were pulsed with Der p 1 in the presence or absence of LAB1 and further cocultured for 2 days with autologous memory allergic T cells. As expected, Der p 1-pulsed DCs, but not unpulsed DCs, enhanced the production of IL-4 and IL-5 by memory T cells. Interestingly, addition of LAB1 to the Der p 1 stimulus reduced the T_H2 cytokine secretion (Fig 4). This inhibitory effect was dose dependent and was only significant at the higher dose of bacteria tested. Moreover, the presence of LAB1 during Der p 1 stimulation favors activated memory T cells to produce higher IFN- γ amounts than with Der p 1 alone. Because these results were correlated with an increase of the percentage of IFN- γ -secreting T cells and a decrease of IL-4-secreting T cells (data not shown), it is suggested that LAB1 might inhibit the already T_H2 -skewed memory T-cell response.

LAB1 effect on T-cell proliferation

When naïve CD4 T cells were cocultured for 5 days with Der p 1-pulsed MD-DCs, only a low percentage of these cells were recruited into divisions, as shown by the

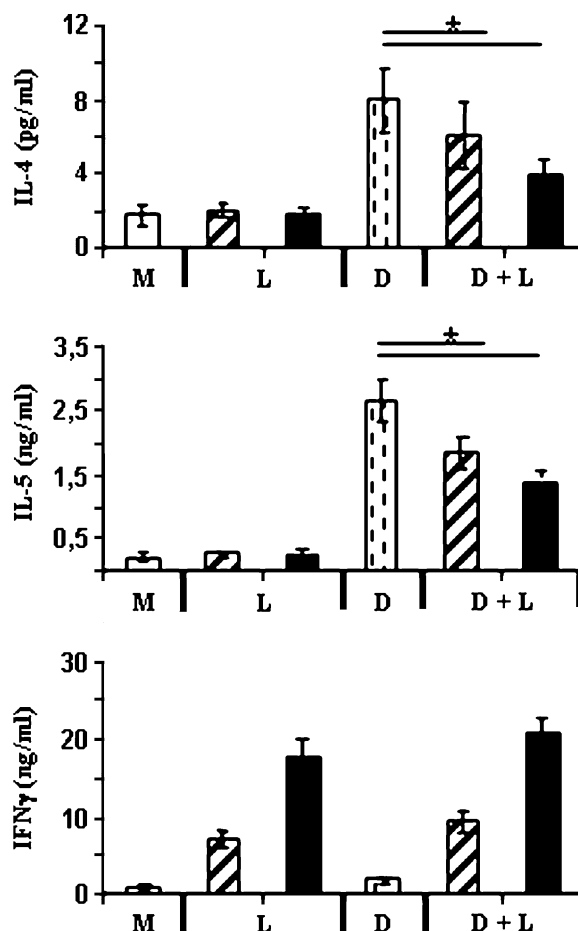


FIG 3. LAB1 effect on the orientation of the T-cell cytokine profile. MD-DCs from allergic donors were pulsed with Der p 1 (D), LAB1 (L), or both (D+L; bacteria/DC ratios, 1:1 [hatched columns] or 10:1 [filled columns]) for 24 hours and cultured with naïve autologous T cells for 5 days. Unpulsed DCs were used as controls (M). IL-4, IL-5, and IFN- γ concentrations were determined by means of ELISA (mean $\pm$ SD values, $n = 6$). *Statistical differences ($P \leq .05$).

decrease in carboxy-fluorescein diacetate succinimidyl ester fluorescence intensity compared with that seen in the unpulsed DCs (Fig 5). Addition of LAB1 to Der p 1 induces a significant proliferation of T-cell response similar to that observed with LAB1 alone. The reactivity of memory T cells was different. Indeed, whereas memory T cells cocultured for 5 days with Der p 1-pulsed MD-DCs proliferated intensively (compared with unpulsed DCs), the addition of LAB1 highly reduced the proliferative response.

LAB1-stimulated DCs generate T cells refractory to a new stimulation with Der p 1 for the production of T_H2 cytokines

Naïve or memory CD4⁺ T cells from allergic patients were first cultured for 5 days with MD-DCs pulsed with Der p 1 in the presence or absence of LAB1, and washed T-cell populations were further incubated with new, fresh

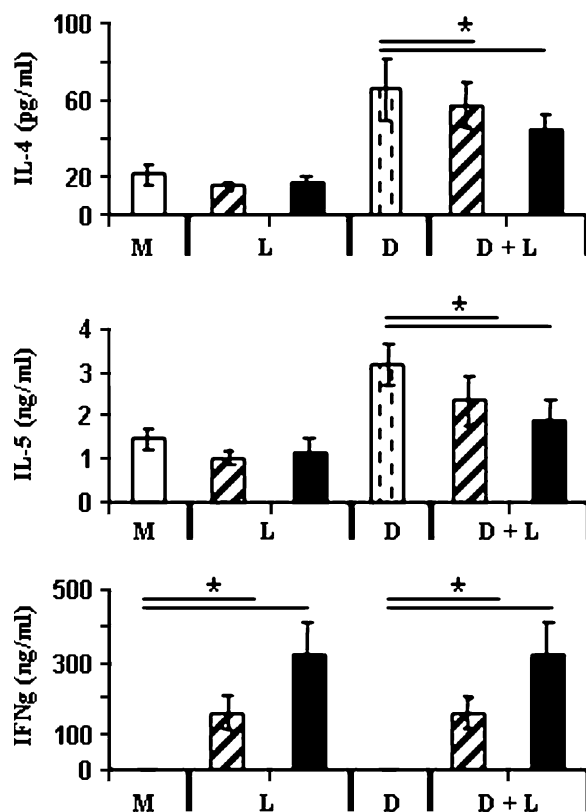


FIG 4. LAB1 effect on an established T_H2 cytokine profile. MD-DCs from allergic donors were pulsed with Der p 1 (D), LAB1 (L), or both (D+L; bacteria/DC ratios, 1:1 [hatched columns] or 10:1 [filled columns]) for 24 hours and cultured with memory autologous T cells for 5 days. Unpulsed DCs were used as controls (M). IL-4, IL-5, and IFN- γ concentrations (mean $\pm$ SD values, $n=6$) were determined at 48 hours. *Statistical differences ($P \leq .05$).

Der p 1-pulsed autologous MD-DCs for 24 hours. In these experimental conditions (Fig 6) naive or memory T-cell populations already stimulated with Der p 1-pulsed DCs were still able to produce large amounts of IL-4 and IL-5. However, T-cell populations previously incubated with DCs pulsed with Der p 1 and LAB1 produced largely less T_H2 cytokine when restimulated with fresh Der p 1-pulsed DCs. Thus the effect of LAB1 on T-cell profile might be considered a lasting effect.

DISCUSSION

Type 2 cytokines, such as IL-4 and IL-5, are involved in the development and maintenance of the allergic immune response. Their selective production is tightly regulated and is highly dependent on the reactivity of DCs to their environment, which in turn contributes to the T-cell polarization process. For the first time, we demonstrated in a model of house dust mite allergy that a lactic acid bacteria (LAB1) reduces the level of T_H2 cytokines produced by naive or memory human T cells through a direct effect on DCs.

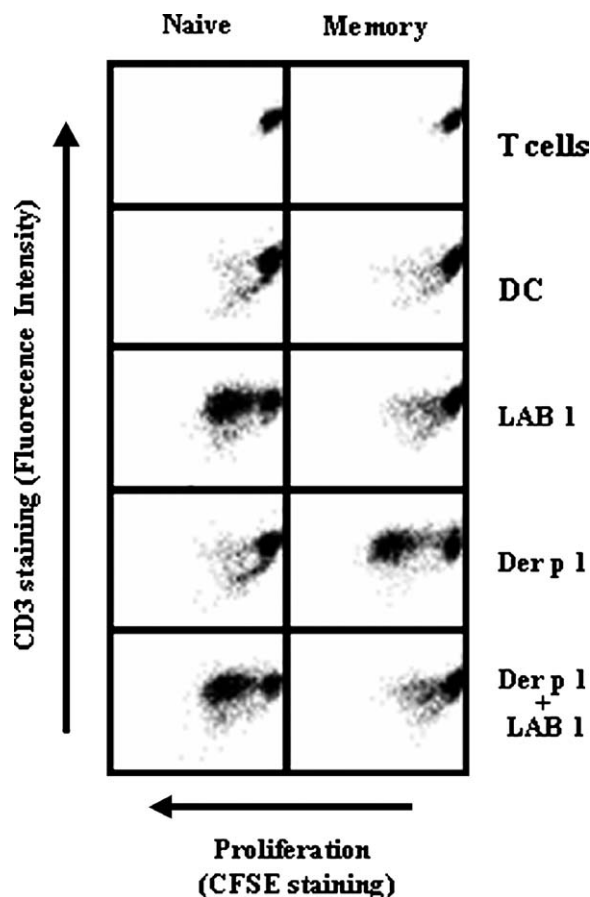


FIG 5. LAB1 modifies the T-cell proliferation induced by Der p 1-pulsed DCs. MD-DCs from allergic donors were pulsed with Der p 1, LAB1, or both for 24 hours and further cultured for 5 days with carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled naive or memory autologous T cells. Unstimulated T cells and unpulsed DCs were used as controls. T-cell division was analyzed by means of flow cytometry. One representative experiment of 4 is shown.

Naive or memory CD4 T cells were stimulated with autologous DCs pulsed in different conditions to provide evidence of the potential effect of LAB1 on T_H2 cytokine production. A previous report showed that MD-DCs from allergic patients favored the T_H2 cytokine production of autologous T cells.¹ Here we demonstrated that when MD-DCs from allergic patients were pulsed with Der p 1 in the presence of LAB1, the stimulated naive and memory T cells decreased their T_H2 cytokine release and increased their IFN- γ production. These results extended our previous observations demonstrating that 4 LAB strains reduced specifically the related allergen-dependent T_H2 cytokine release from PBMCs of allergic patients. A gram-negative bacteria, *Escherichia coli* TG1, used as a control had no effect on T_H2 cytokine production.⁷ Other studies have reported IFN- γ production upregulation in mice injected with yogurt-derived lactobacilli.⁸ IFN- γ is known to downregulate T_H2 responses by antagonizing IL-4,⁹ which might explain why, in our *in vitro* model, the presence of LAB1 was always associated with the

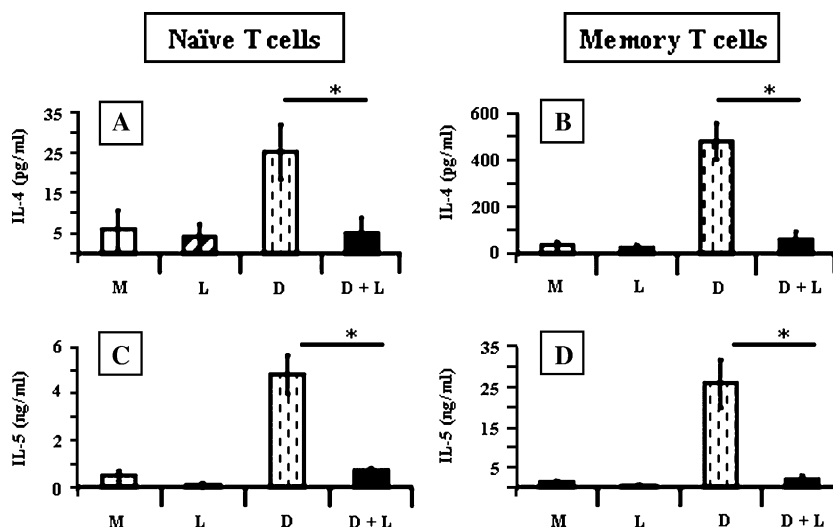


FIG 6. LAB1-stimulated DCs generate T cells refractory to a new Der p 1 stimulation. MD-DCs from allergic patients were incubated for 24 hours with LAB1 (L), Der p 1 (D), both stimuli (D+L), or medium alone (M) and with memory or naïve T cells for 5 days. Finally, in each condition washed T cells were cocultured with Der p 1-pulsed MD-DCs for 24 hours. IL-4 and IL-5 concentrations from 4 experiments are represented.

reduction of T_H2 cytokine release. In our case the $IFN-\gamma$ production enhancement was related to the increase in IL-12 production observed when MD-DCs from allergic patients were pulsed by Der p 1 in the presence of LAB1. Reports in human subjects and mice have emphasized the increased IL-12 production in leukocyte cultures incubated with LAB strains.^{10,11} However, the source of IL-12 in LAB-treated cultures was not clearly identified. Our data suggest that DCs might be one key cell type. Moreover, IL-12 is known to limit the establishment and maintenance of T_H2 -type responses,^{12,13} mainly by enhancing $IFN-\gamma$ production and by providing an effective deviation signal during the early differentiation of T_H0 cells.¹⁴ Thus because LAB1 led to IL-12 production, it might contribute to switching the balance from an established T_H2 response to a more pronounced T_H1 response.

Attempts to evaluate in allergic patients the effect of LAB1 on the specific T-cell proliferation led to an apparent unexpected dichotomy for naïve and memory T cells. Whereas naïve T cells exhibited a weak proliferative response to Der p 1-pulsed DCs, a higher T-cell proliferation was observed with LAB1-treated MD-DCs. T-cell proliferation requires at least 2 signals, one through contact of antigen-MHC with T-cell receptor and another through interaction between costimulatory molecules.^{15,16} Thus the variable degree of DC maturation might explain the differences observed in T-cell proliferation. Indeed, in response to Der p 1, DCs expressed quite low levels of costimulatory molecules compared with LAB1-pulsed DCs, which expressed intense upregulation of their surface markers (CD80, CD86, CD83, and HLA-DR). However, a polyclonal T-cell activation by LAB1 cannot be excluded, as already reported for other products derived from gram-positive bacteria.^{17,18} Precise characterization

of the mechanism leading to the expansion of the naïve T-cell population is currently under investigation. Nonetheless, an apparent relationship was evidenced between the emergence of this LAB1-dependent T-cell population and the increase in $IFN-\gamma$ production. In contrast, concerning the memory T cells, their intensive proliferation observed with Der p 1-pulsed DCs was highly reduced in the presence of LAB1. Because memory T cells have already been primed by the antigen, their triggering requirement was different from that necessary for naïve T-cell stimulation.¹⁹⁻²¹ Thus although additive experiments are in progress, the mechanisms by which LAB1 induced decreased memory CD4 T-cell proliferation might be the result of a combined effect of the LAB on the uptake of Der p 1 by DCs, on the level of $IFN-\gamma$ production, and on TLR regulation. Indeed, TLRs, key elements in pathogen recognition, are involved in DC maturation.²² Both TLR2 and TLR4 were implicated in the recognition of bacteria wall products of gram-positive²³ and gram-negative²⁴ bacteria. However, preliminary experiments showed that the low TLR2 and TLR4 expression on the Der p 1-pulsed DC surface was not significantly modified in the presence of LAB1 (data not shown).

Finally, the progressive action of T regulatory cells cannot be excluded. T cells first incubated with Der p 1-pulsed MD-DCs and then restimulated with fresh Der p 1-pulsed MD-DCs enhanced the production of T_H2 cytokines. T cells previously incubated with MD-DCs pulsed with Der p 1 and LAB1 and then with Der p 1-pulsed MD-DCs became refractory to a new stimulation with Der p 1 and produced very low amounts of IL-4 and IL-5. Interestingly, a large part of T cells induced by MD-DCs pulsed with Der p 1 and LAB1 appeared to be $CD4^+CD25^+$ T cells secreting IL-10 (data

not shown). Recently, some LAB strains were shown to increase the levels of IL-10 *in vivo* and *ex vivo*.²⁵ IL-10 has been described to reduce T_H2 cell differentiation and expansion.²⁶ The IL-10 release could participate in the inhibition of the T_H2 cytokine production induced by Der p 1. Other mechanisms provided by cellular contact (through molecules, such as CD152) with this T cell might also be involved. A further characterization of these cells and their involvement in the LAB1 effect still remains to be addressed.

In conclusion, our results suggest that LAB1 might switch the established T_H2 response in allergic patients toward a long-lasting T_H1 response and might therefore represent a new therapeutic strategy for the treatment of allergic diseases.

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