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Original article

Immunomodulatory properties of *Lactobacillus plantarum* and its use as a recombinant vaccine against mite allergy

Background: Selected lactic acid bacteria were reported to prevent atopic dermatitis and experimental asthma but the mechanisms of their immunomodulatory effects are not fully elucidated. In this study, the signaling pathways triggered by *Lactobacillus plantarum* NCIMB8826 were investigated and the potential use of this strain producing a variant of the mite allergen Der p 1 as live vaccine vehicle was evaluated.
Methods: Mouse bone marrow-derived dendritic cells were stimulated with wild-type or a *L. plantarum* teichoic acid mutant to evaluate the secretion of cytokines. A recombinant *L. plantarum* expressing Der p 1 was engineered, its *in vitro* immunomodulatory properties were characterized and its prophylactic potential was evaluated in a Der p 1-sensitization murine model.
Results: Mouse dendritic cells stimulated by *L. plantarum* triggered the release of interleukin-10 (IL-10), IL-12 p40, IL-12 p70 and tumor necrosis factor-alpha (TNF-α). IL-12 p40 secretion was dependent on nuclear factor-κB (NF-κB), mitogen-activated protein (MAP) kinases, Toll-like receptor 2 (TLR2), TLR9 and on the bacterial teichoic acid composition. Recombinant *L. plantarum* producing Der p 1 exhibited similar immunostimulatory properties as wild-type. Prophylactic intranasal pretreatment of mice with this recombinant strain prevented the development of the typical Th2-biased allergic response by a drastic reduction of specific IgE and the induction of protective allergen-specific IgG2a antibodies. Moreover, both wild-type or recombinant *L. plantarum* reduced airway eosinophilia following aerosolized allergen exposure and IL-5 secretion upon allergen restimulation.
Conclusion: By combining both Th1-type immunostimulatory properties and an efficient allergen delivery capacity, recombinant *L. plantarum* producing Der p 1 represents a promising vaccine against house dust mite allergy.

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Key words: Der p 1; recombinant *Lactobacillus plantarum;* Toll-like receptor; vaccination.

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Commensal bacteria display health promoting effects by driving the development of the mucosal immune system. Their immune modulation activities are not only essential for the activation of tolerogenic mechanisms to foreign harmless antigens (1) but also for the maintenance of intestinal homeostasis (2). Lactic acid bacteria (LAB) are Gram-positive micro-organisms which colonize very early the gut of neonates. Immunomodulatory properties of LAB were demonstrated in the treatment of inflammatory disorders like pouchitis (3) and ulcerative colitis (4). To date, the precise mechanism of LAB-induced immunomodulation is not fully elucidated although it was reported that these probiotics can directly but differentially modulate dendritic cells (DC) maturation and induce cytokine secretion through toll-like receptors (TLR) including TLR2 (5–7). Some studies evidenced the role of LAB in peripheral T-cell hyporesponsiveness (8), and promotion of regulatory T-cell development through DC modulation (9). Consequently, LAB could play a beneficial role in the prevention or treatment of the Th2-biased allergic response. Epidemiological studies have reported the positive influence of lactobacilli and bifidobacteria microflora (10) on allergy incidence. Preclinical studies in murine models (11, 12) as well as clinical trials (13) have highlighted the beneficial effects of LAB, such as *Lactobacillus plantarum* NCIMB8826 (6), in the prevention of allergy.

The present study aimed to further characterize the immunomodulatory properties of *L. plantarum* NCIMB8826 at the level of the signaling pathways activated in DC. Since this bacterial strain displays a strong anti-Th2 capacity, we engineered a recombinant *L. plantarum* producing the major house dust mite allergen Der p 1 for mucosal antigen delivery purpose and evaluated the prophylactic effects of this putative *L. plantarum*-based live mucosal vaccine.

Methods

Animals

Female Balb/c and C57BL/6 (B6) mice (6 weeks old) were obtained from Harlan (Horst, The Netherlands). TLR2-, TLR9- and MyD88-deficient mice with B6 background (6 weeks old) were kindly gifted from Dr S. Akira (RIMD, Osaka University, Japan) and Dr M. Moser (IBMM, ULB, Belgium). Animal care and experimental procedures were carried out in accordance with local institutional guidelines (laboratory license No. LA 1500474).

Bacterial strains and growth conditions

Lactobacillus plantarum NCIMB8826 (WT), *L. plantarum* EP007 (*dlt*$^-$), were grown as previously described (14, 15). For *in vitro* stimulation, fresh medium was inoculated with *L. plantarum* at optical density (OD) of 600 nm (OD_{600}) of 0.15, harvested 3 h later by centrifugation (11 min at 2500 ***g***) and prepared for storage as described before (14). The endotoxin content of the bacterial preparations was below 5.8 EU/ml as estimated by limulus assay (QCL 1000 chomogenic LAL endpoint essay, Lonza, Walkersville, MD, USA). Chromosomal DNA of *L. plantarum* was isolated according to the method of Ferain et al. (16).

Construction of a *Lactobacillus plantarum* recombinant strain producing Der p 1

The MBP-ProDer p 1 coding sequence from pNIV4854 plasmid (unpublished results) was amplified by polymerase chain reaction (PCR) using the following primers: 5′ CTAGTCATGAAAACT-GAAGAAGGTAAACTGG 3′ (*Bsp*HI restriction site underlined, forward) 5′ GCTCTAGACTCGAGGGGATCCTTTACAGG 3′ (*Xba*I restriction site underlined, reverse). The amplified deoxyribonucleic acid (DNA) fragment, subcloned into the pMOSblue cloning vector (GE Healthcare, Piscataway, NJ, USA), was digested by *Bsp*HI and *Xba*I and cloned into the pNZ8037 expression vector (17) restricted with *Nco*I and *Xba*I. The resulting plasmid (pMEC240) contains the MBP-ProDer p 1 coding cassette under the control of the nisin-inducible P_{nisA} promoter and was introduced in *L. plantarum* NCIMB8826 Int-1 strain (18) by electroporation. To generate non-producing control strain, *L. plantarum* NCIMB8826 Int-1 was electroporated with empty pNZ8037. Induction of MBP-ProDer p 1 production by *L. plantarum* NCIMB8826 Int-1 was performed as previously described (18) with some modifications. Optimal parameters for the recombinant MBP-ProDer p 1 expression were assessed by immunoblotting assay (data not shown). Maximal MBP-ProDer p 1 production is reached by induction of recombinant *L. plantarum* production at an OD_{600} of 0.15 and with the following culture parameters: 37 °C, 50 ng/ml nisin, 3 h of induction.

Antigens

Natural Der p 1 and recombinant ProDer p 1 were purified from whole *Dermatophagoides pteronyssinus* cultures and Chinese hamster ovary spent culture medium, respectively, as previously described (6).

Preparation and activation of mouse bone marrow-derived dendritic cells

Mouse bone marrow-derived dendritic cells (BMDC) were prepared as previously described (19) with minor modifications. Briefly, after red cell lysis, bone marrow cells isolated from femurs and tibias were seeded at 1.2×10^6 cells/ml for 9 days in Rosewell Park Memorial Institute (RPMI) 1640 culture medium containing 10% fetal bovine serum (FBS), 150 μg/ml gentamycin and 20 ng/ml mouse rGM-CSF (kind gift of K. Thielemans, VUB, Brussels, Belgium). Cells were collected on day 9. The purity of BMDC preparations was above 97% as estimated by CD11c staining. BMDC (10^6 cells/well) were incubated with 10×10^6 colony forming units (CFU) of bacteria or with purified *L. plantarum* chromosomal DNA (6.25–25 μg/ml) in fresh BMDC medium for 24 h. As controls, BMDC were also incubated with PAM2CysSerLys4 (100 ng/ml, InvivoGen, San Diego, CA, USA) or CpG ODN 1826 (1 μg/ml, Eurogentech, Seraing, Belgium). When appropriate, BMDC were pretreated at 37 °C for 1 h with mitogen-activated protein kinase (MAPK) (U0126, 25 μM; SB203580, 25 μM; SP6001125, 25 μM) or NF-κB (MG132, 25 μM; BAY-11–7082, 25 μM) specific inhibitors. In these experimental conditions, BMDC were stimulated with 100×10^6 CFU of bacteria during 6 h. Culture supernatants of stimulated BMDC were collected and stored at −80 °C until use. Mouse IL-12 p40, IL-12 p70, IL-10, TNF-α were assayed in culture supernatants by enzyme-linked immunosorbent assay (ELISA) using BD OptiEIA kits (BD, Erembodegem, Belgium). For cell surface marker analysis, BMDC were labeled for 30 min at 4 °C with phycoerythrin (PE)-conjugated anti-CD40, CD80, CD86 or Class II MHC ($I\text{-}A^d$) monoclonal antibody (BD) before flow cytometry analysis (FACS Canto, BD, Erembodegem, Belgium).

Human cell transfection and luciferase assay

Wild-type human embryonic kidney 293 (HEK 293) or HEK 293 T2Y cells (20) (HEK 293 cells stably expressing human TLR2 receptor fused to yellow fluorescent protein (YFP) kindly provided by Dr D.T. Golenbock, University of Massachussets Medical School, USA). Cells (3×10^5 cells/ml) were then transiently transfected with NF-κB reporter plasmid pTK-4xNF-κB-luc (21) mixed with Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer instructions (1 μg plasmid/3 μl Fugene 6). Cells were then stimulated with bacteria at indicated concentrations, TNF-α (10 ng/ml) or PAM2CysSerLys4 (100 ng/ml) for 24 h. Cells were lysed using passive lysis buffer (Promega, Madison, WI, USA) and luciferase activity of the cellular lysate was measured with Luciferase Assay System (Promega). Luciferase activity was normalized to total protein content measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Human IL-8 was assayed in culture supernatants by ELISA using BD OptiEIA kits (BD, Erembodegem, Belgium).

Immunoblotting assay

Bacterial cell extracts were analyzed in western blotting using a mouse polyclonal serum raised against ProDer p 1 (1 : 2000) or an anti-MBP mouse monoclonal antibody (NEB, 1 : 2000) followed by horseradish peroxidase-conjugated horse anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA, 1 : 2000).

Vaccination protocol and measurement of allergen-specific response

Balb/c mice (n = 9–10) were intranasally (i.n.) pretreated on days 0–3 and 7–10 with 10^9 CFUs (15 μl/nostril) of either recombinant or control *L. plantarum*, or saline (Fig. 5). Seven days after the last

vaccination (day 17), animals were subsequently intraperitoneally sensitized for 3 weeks at weekly intervals with 2 μg of natural Der p 1 (day 17, 24 and 31) formulated with alum (ratio allergen/adjuvant of 1/50). Sera were collected on day 38. To induce airway inflammation, mice were challenged 10 days after the last sensitization by exposure to aerosolized crude *D. pteronyssinus* extracts (containing 10 μg/ml Der p 1) over a 30-min period on days 43, 44, 45 and 47. Anti-ProDer p 1 IgG1 IgG2a and IgE antibody assays and bronchoalveolar lavage (BAL) fluids analysis were performed as previously described (22). For T-cell proliferation, spleens were collected individually whereas mediastinal lymph nodes were pooled from two mice. Cells were then seeded in 96-well U-bottomed tissue culture plates at 2×10^5 or 3×10^5 cells/well for lymph node and spleen cells, respectively. The Der p 1-specific T-cell proliferative responses were measured as previously described (22). Levels of interferon-γ (IFN-γ) and IL-5 production were assessed as described before (22).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Student's *t*-test for comparison between groups.

Results

Stimulation of bone marrow-derived dendritic cells by *Lactobacillus plantarum* depends on TLR2 and TLR9 and is related to lipoteichoic acid composition

To define the mode of action of *L. plantarum* as a potential vaccine adjuvant, we investigated its effects on mouse BMDC. To analyze whether *L. plantarum* stimulates BMDC through TLR9 activation, we generated BMDC from mice deficient in TLR9 which specifically recognized unmethylated CpG dinucleotides from bacterial DNA. We compared the IL-10 and IL-12 p40 production by *L. plantarum*-stimulated BMDC from WT, MyD88-, TLR2- and TLR9-deficient mice (Fig. 1A–D). Whereas the production of IL-10 and IL-12 p40 by BMDC was blunted in MyD88-deficient BMDC, DC generated from TLR2- and TLR9-deficient mice were significantly less responsive to *L. plantarum* stimulation than BMDC generated from WT mice (Fig. 1A–D). These results showed the total MyD88-dependence and the partial TLR2 dependence of IL-10 and IL-12 p40 production triggered by *L. plantarum* but highlighted also the importance of TLR9 in some cell activation pathways induced by this LAB. Indeed *in vitro* experiments carried out with *L. plantarum* chromosomal DNA in TLR9 deficient mice demonstrated that the production of IL-12 p40 but not IL-10 by BMDC was dependent on TLR9 pathway (Fig. 1C and D).

To confirm that *L. plantarum* uses TLR2 to exert its stimulatory action, we evaluated its effects on human HEK 293 cells stably expressing human TLR2 and co-transfected with a NF-κB-dependent reporter plasmid. Following stimulation of HEK 293 cells with different concentrations of *L. plantarum*, NF-κB activation was measured by luciferase essays and IL-8 secreted in supernatants was determined (Fig. 1E and F). In WT HEK 293 cells, *L. plantarum* used at 100×10^6 CFU induced NF-κB production resulting probably from a TLR-independent mechanism, the nature of which remaining undetermined. The TLR2 overexpression in HEK 293 cells induced a more pronounced NF-κB activation and IL-8 secretion by *L. plantarum* (Fig. 1E and F).

Finally, to evaluate the implication of lipoteichoic acid (LTA) in the intensity of TLR2-dependent-BMDC stimulation, we used a *L. plantarum dlt*$^-$ mutant resulting from a defect in D-alanylation of LTA. This mutant strain was a much less potent cytokine inducer from WT BMDC than the WT bacteria (Fig. 1A and B). On the other hand, this mutant strain displayed a similar TLR2, TLR9 and MyD88 dependence (Fig. 1A). Using HEK luciferase assays, we showed that weaker amounts of NF-κB and IL-8 were induced through TLR2 by *dlt*$^-$ mutant (Fig. 1E and F).

Taken together, our present results suggested that the immunomodulatory activity of *L. plantarum*, at least mediated by LTA and chromosomal DNA, is clearly dependent on TLR2 and TLR9 signaling pathways.

Interleukin-12 p40 production induced by *Lactobacillus plantarum* stimulation involves nuclear factor-κB and mitogen-activated protein kinase pathways.

To assess the role of MAPKs and NF-κB activation on the production of IL-12 p40 from mouse BMDC to *L. plantarum* response, cells were incubated with specific inhibitors of Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) MAPK as well as NF-κB pathways inhibitors (Fig. 2). Pretreatment of BMDC with JNK (SP600125), p38 (SB203580) and NF-κB inhibitors (IKB-α phosphorylation BAY-11-7082 and proteasome MG132 inhibitors) during 1 h drastically reduced *L. plantarum*-induced IL-12 p40 production. The reduction of IL-12 p40 secretion was also observed by using the MEK1/2 inhibitor U0126, but to a lesser extent. The cytokine production induced by Pam2CysSK4 (TLR2 agonist) followed a NF-κB and strict JNK signaling dependence. These data suggest that activation of JNK, p38 and NF-κB pathways is essential for IL-12 p40 production in *L. plantarum*-treated BMDC.

Development of a recombinant *Lactobacillus plantarum* strain producing Der p 1

Because *L. plantarum* exhibits immunomodulatory properties *in vitro* and *in vivo*, the potential use of recombinant *L. plantarum*-based live vaccine vehicle for mucosal delivery of mite allergen Der p 1 was evaluated. Thus, the coding cassette for ProDer p 1, the precursor form of Der p 1, was cloned in the nisin-inductible expression

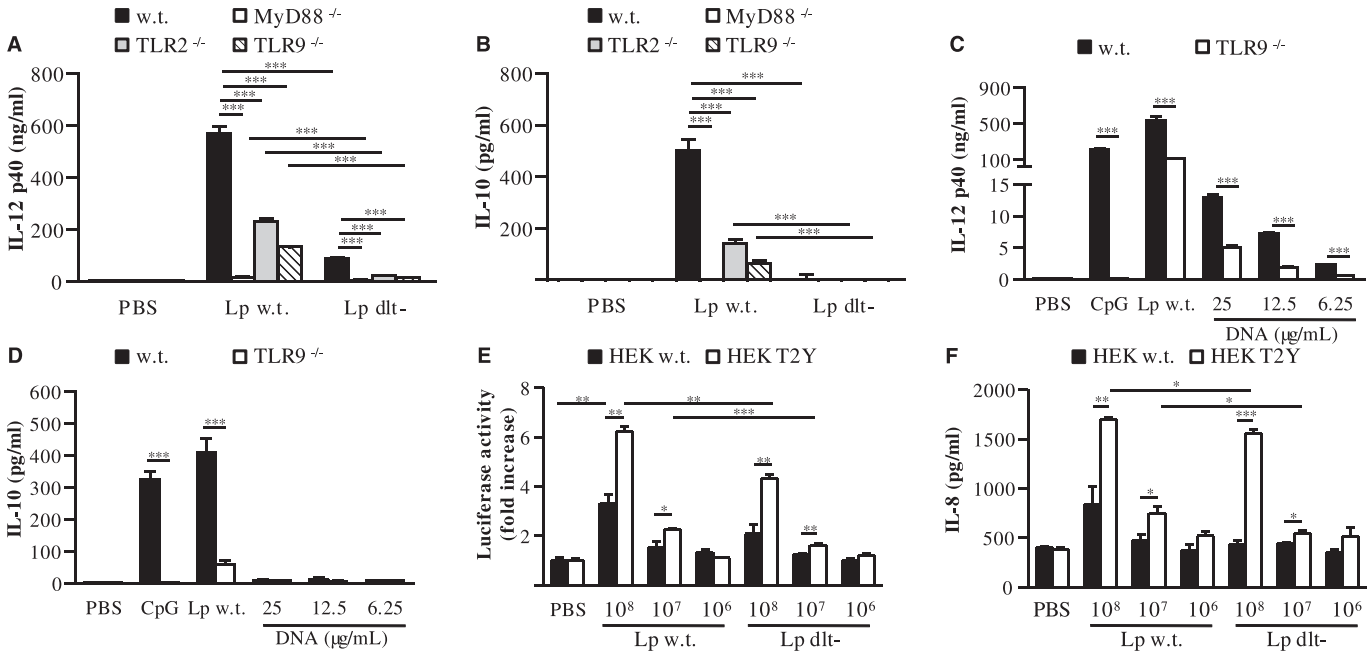

Figure 1. Stimulation of BMDC by *L. plantarum* is related to LTA composition and depends on TLR2 and TLR9. BMDCs generated from WT, TLR2-, TLR9- or MyD88-deficient mice were pulsed for 24 h with saline (PBS), WT (Lp w.t.: 10×10^6 CFU) or *dlt*- (Lp dlt-: 10×10^6 CFU) *L. plantarum*, CpG ODN 1826 (1 μg/ml) or *L. plantarum* DNA (A, B, C, D). IL-12 p40 (A, C) and IL-10 (B, D) concentrations are shown as mean ± SEM. One representative experiment out of 3 is shown. WT (HEK w.t.) or T2Y (HEK T2Y) HEK 293 cells, transiently transfected with a NF-κB reporter plasmid encoding firefly luciferase, were stimulated for 24 h with 1–100×10^6 CFU of WT (Lp w.t.) or *dlt*- (Lp dlt-) *L. plantarum* (E, F). Luciferase activity induction (E) and IL-8 concentrations (F) are shown as mean ± SEM. One representative experiment out of 3 is shown. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (deficient mice compared to WT, A, B, C, D. *dlt*- bacteria compared to WT, A, B, E, F. Stimuli compared to PBS, E, F. HEK T2Y compared to HEK WT, E, F).

vector pNZ8037 to induce intracellular allergen production in *L. plantarum*. Under our experimental conditions, no production of ProDer p 1 could be detected after addition of nisin in the culture medium (data not shown). In contrast, we successfully produced intracellularly ProDer p 1 fused to the N-terminus of *Escherichia coli* MBP (Fig. 3, lane 5). Nisin induction resulted in the production of a protein with the expected molecular mass (73 kDa) which was recognized in western blotting by murine polyclonal antibodies directed to ProDer p 1 as well as by an anti-maltose bonding protein (anti-MBP) monoclonal antibody. Blot intensity comparison using serial dilutions of purified MBP-ProDer p 1 from *E. coli* estimated that 10^9 CFU of *L. plantarum* recombinant strain produced 10 ± 2.5 μg of recombinant antigen after nisin induction (20% of total intracellular protein content) (data not shown).

Recombinant *Lactobacillus plantarum* maintained its capacity to activate mouse dendritic cells

We compared the ability of wild-type and recombinant *L. plantarum* strains to induce dendritic cell maturation as well as cytokine production in mouse BMDC. Compared to stimulation with bacterial lypopolysaccharide (used as positive control), incubation with control or recombinant *L. plantarum* resulted in a similar and significant increase of the surface expression of CD40, CD86 and to a lesser extend of MHC class II molecule I-A^d and CD80 on BMDC (Fig. 4A). The genetic transformation of *L. plantarum* did not modify its capacity to stimulate cytokine secretion by BMDC as judged by a similar IL-12 p40, IL-12 p70, IL-10, and TNF-α production (Fig. 4B–E).

Prevention of experimental house dust mite allergy by recombinant *Lactobacillus plantarum* intranasal application

The prophylactic potential of the recombinant *L. plantarum* strain producing Der p 1 was assessed in a Der p 1 sensitization murine model (Fig. 5). Der p 1-sensitized mice developed a strong Th2-based allergen-specific response characterized by high specific IgE and IgG1 titers (Fig. 5A and B). Intranasal pretreatment with Der p 1 expressing *L. plantarum* impaired this allergic response as significant specific IgG2a (Fig. 5C) production together with a drastic reduction of the allergen-specific IgE antibodies was elicited. This immunomodulation was not observed with the control strain. On the other hand, the IgG1 specific response was not modulated by any

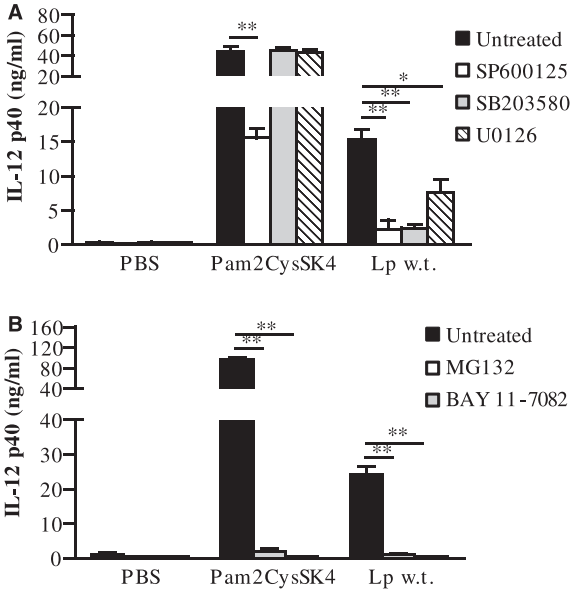

Figure 2. IL-12 p40 production induced by *L. plantarum* stimulation involves NF-κB and MAPK pathways. BMDC were incubated with JNK, p38 or MEK1/2 (SP600125, SB203580, U0126; A) or NF-κB pathway inhibitors (MG132, BAY 11-7082; B) before stimulation (6 h) by WT *L. plantarum* (Lp w.t.: 10^8 CFU), saline (PBS), Pam2CysSK4 (100 ng/μl). IL-12 p40 concentrations are shown as mean ± SEM. One representative experiment out of 3 is shown. $*P \leq 0.01$, $**P \leq 0.001$ (inhibitor treatment compared to sham treatment, A, B).

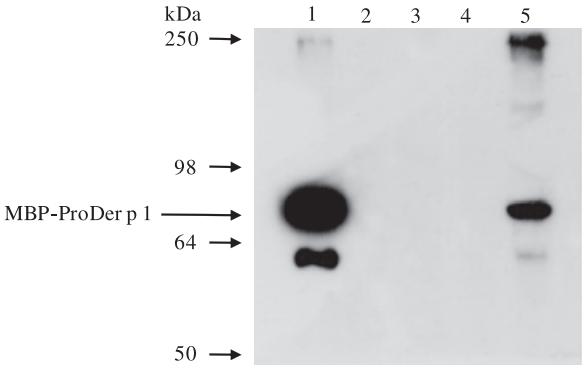

Figure 3. Production of recombinant MBP-ProDer p 1 in *L. plantarum*. Whole bacterial cell lysates were analyzed by Western blot with an anti-ProDer p 1 polyclonal antibody. Lane 1: purified MBP-ProDer p 1. Control (NCIMB8826 Int-1 [pNZ8037], lanes 2, 3) or recombinant *L. plantarum* (NCIMB8826 Int-1[pMEC240], lanes 4, 5) were grown in the presence (lanes 3, 5) or absence (lanes 2, 4) of nisin. Band lower than 73 kDa represents protein degradation.

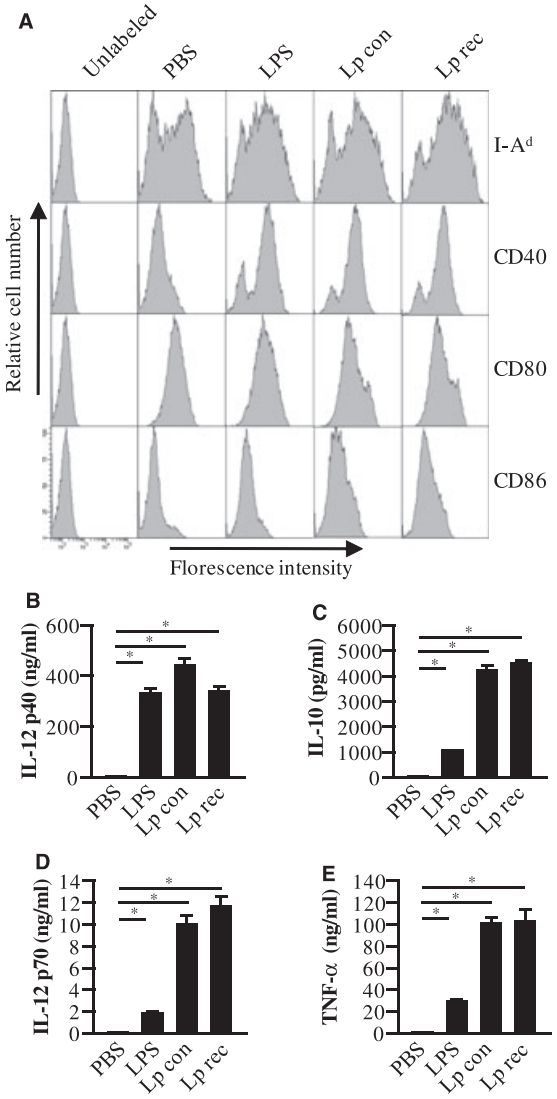

Figure 4. Recombinant *L. plantarum* maintained its capacity to activate DC. BMDC were pulsed for 24 h with saline (PBS), LPS (LPS), control (NCIMB8826 Int-1 [pNZ8037], Lp con) or recombinant (NCIMB8826 Int-1[pMEC240], Lp rec) *L. plantarum*. BMDCs were analyzed by means of cytometry for I-A^d, CD40, CD80, CD86 expression (A). One representative experiment out of 3 is shown. Cytokine concentrations of (IL-12 p40, IL-10, IL-12 p70, TNF-α; B, C, D, E) are plotted as mean ± SEM. One representative experiment out of 3 is shown. $*P \leq 0.001$ (stimuli compared to PBS and Lp rec compared to Lp con, B, C, D, E).

form of *L. plantarum*. These results showed that pretreatments with the allergen producing LAB prevent the allergic response.

We next analyzed IL-5 and IFN-γ secretions by allergen-restimulated spleen cells or lung draining lymph node mononuclear cells (Fig. 5D and E). Pretreatment with wild-type or recombinant *L. plantarum* strains resulted in a marked alteration in IL-5 secretion by spleen or mediastinal lymph nodes cells. However, the reduction was most pronounced in mice immunized with the recombinant strain. No significant effect on IFN-γ secretion was observed. Bronchoalveolar lavage fluid analysis was performed to examine the effects of

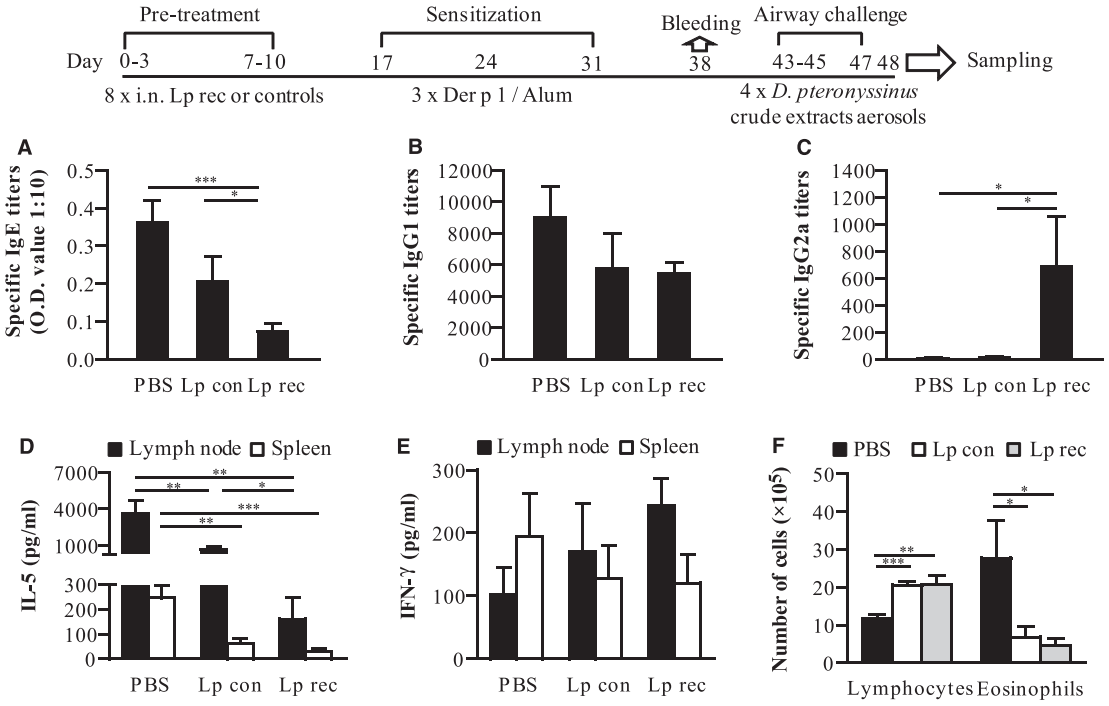

Figure 5. Prevention of experimental house dust mite allergy by Der p 1 expressing recombinant *L. plantarum* intranasal vaccination. Mice (9–10 per group of treatment) were pretreated with saline (PBS), control (Lp con) or recombinant (Lp rec) *L. plantarum*, sensitized to Der p 1 and challenged. Antibody titers (IgE, A; IgG1, B; IgG2a, C), mediastinal lymph nodes cells and splenocytes (IL-5, D; IFN-γ, E) cytokine production, airway cellular infiltrates (F) were plotted as mean ± SEM. One representative experiment out of 3 is shown. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ (comparisons between treatments).

intranasal probiotic application on the development of allergen-induced airway inflammation (Fig. 5F). Pretreatments with both *L. plantarum* strains significantly decreased eosinophil content in BAL fluids but did not modulate the influx of neutrophils, monocytes or macrophages in the airway (data not shown). These results demonstrated that the immune response induced by allergen producing LAB has beneficial effects on allergen-induced airway inflammation.

Discussion

In the present study, we extended our previous observations related to the immumodulatory properties of *L. plantarum* by the use of dendritic cells generated from TLR2-deficient mice and HEK 293 cells expressing human TLR2 (6). We confirmed that *L. plantarum* stimulates innate immunity through at least TLR2. Since TLR2 activates NF-κB and MAPK, leading to the production of cytokines involved in innate immunity (23), we studied the impact of *L. plantarum* on these signaling pathways. Luciferase assays not only underlined the prominent role of TLR2 signaling in the *L. plantarum*-induced dendritic cell activation but clearly showed that this probiotic induced NF-κB activation through TLR2. Preincubation with specific NF-κB signaling inhibitors further evidenced the critical role of NF-κB in IL-12 p40 production in response to *L. plantarum* activation in BMDC. In contrast, a recent report demonstrated that another *L. plantarum* strain (ATCC 8014) does not activate the NF-κB pathway but inhibits TNF-α-induced NF-κB translocation to the nucleus and IκB-α degradation (24). It must be pointed out that the strain-specific dependence of the immunoregulatory effects of lactic acid bacteria was well documented (5). Whereas the MAPK activation by *Lactobacillus acidophilus* or *Lactobacillus casei* strains has been reported (25, 26), such stimulation of MAPK signaling by *L. plantarum* was not yet evidenced. The data obtained with specific MAPK inhibitors strongly emphasized the importance of coactivation of p38, JNK and to a lesser extent of ERK signaling pathways in the effect of *L. plantarum* on IL-12 p40 production

The partial reduction of IL-12 p40 and IL-10 production in *L. plantarum*-treated DC from TLR2-deficient mice suggested the involvement of other MyD88-dependent TLRs. Therefore, we next evaluated the role of TLR9 in the immunomodulation of *L. plantarum* as Toll-like receptor 9 signaling was shown to mediate the anti-inflammatory effects of other probiotics in murine experimental colitis (27). Using purified *L. plantarum*

chromosomal DNA as well as BMDC from TLR9-deficient mice, we not only highlighted the importance of TLR9 signaling in the effect of *L. plantarum* on DC function but clearly evidenced the contribution of the bacterial DNA to the immunomodulatory properties of the bacteria.

Recently, several reports showed that the proinflammatory capacity of highly purified and biologically active LTAs from probiotics is TLR-2-dependent (14, 28). By comparing the immunostimulatory properties of *L. plantarum* with those of a cell-wall mutant deficient in D-alanylation of LTA (*dlt*$^-$ mutant (14, 29)) on mouse TLR-deficient BMDC and human HEK 293 TLR2, we observed that both *L. plantarum* strains show a similar TLR2 and TLR9 dependence. However, our data differ from a previous report which demonstrated no significant TLR-2 dependence of the *dlt*$^-$ mutant cells (14). It should be noted that the *L. plantarum dlt*$^-$ mutant was collected at the end of the stationary phase (48 h) whereas the corresponding bacteria used in the present study were harvested in exponential phase (after 3 h). Morphological changes and especially autolysis of the *dlt*$^-$ mutant was observed in stationary phase but to a lesser extent during the exponential phase and this could significantly influence the immunomodulatory properties of the *dlt*$^-$ mutant (29). Moreover, the use of BMDCs from wild-type and TLR2 deficient mice instead of bone marrow precursor cells might explain difference in cell reactivity.

In view of these *in vitro* results and our previous data on the prevention of house dust mite allergy in mice after the co-administration of the mite allergen Der p 1 with the NCIMB8826 strain (6), we hypothesized that *L. plantarum* could represent an efficient live vaccine for Der p 1 delivery.

Consequently, we engineered a recombinant strain of *L. plantarum* NCIMB8826 producing the precursor form of Der p 1 (ProDer p 1) in fusion with the MBP. In contrast to the Bet v 1 (15) or Der p 5 (30) allergen, the fusion partner was critical for recombinant Der p 1 expression as no expression was detected for mature Der p 1 or ProDer p 1 alone (data not shown). This absence of expression could be more likely explained by the cysteine proteinase-dependent cytotoxicity of mature Der p 1 and/or by an inappropriate refolding of ProDer p 1 in *L. plantarum*.

As we first demonstrated that the Th1-biased cytokine profile induced following the probiotic stimulation of BMDC is not influenced by the recombinant Der p 1 expression, we next evaluated the efficacy of the recombinant *L. plantarum* strain producing Der p 1 in the prevention of house dust mite allergy using a Der p 1-sensitization murine model. The intranasal route was chosen for mucosal delivery of Der p 1 by recombinant *L. plantarum* for several reasons: (1) Systemic and mucosal responses (even at distant sites) can be easily obtained by intranasal antigen applications (31). (2) Compared with intranasal vaccinations, oral immunizations with recombinant *L. plantarum* expressing Bet v 1 or wild-type strain with Der p 1 induced a weak and nonprotective immune response (unpublished results). (3) Intranasal vaccinations of mice were shown to be fully innocuous (data not shown), similarly to oral administration. Our study demonstrated that intranasal prophylactic vaccination with this recombinant LAB expressing Der p 1 inhibited the development of Der p 1-specific allergy as evidenced by very weak specific IgE titers and the absence of airway eosinophilia. The typical Th2-biased allergic immune response was indeed prevented by the induction of a Der p 1-specific Th1 profile characterized by high IgG2a titers, very low IL-5 secretion upon splenocyte or draining lymph node restimulation. In contrast, wild-type or recombinant *L. plantarum* did not modulate the specific IgG1 response more likely because of the use of alum, a strong IgG1 inducer adjuvant, for the Der p 1 sensitizations. Although the blocking activity of the induced specific IgG2a remains to be elucidated, it is tempting to speculate that these IgG2a could compete with IgE binding to Der p 1. It is also interesting to note that *L. plantarum* wild-type alone had no effect on the Der p 1-specific IgE but was able to reduce IL-5 secretion and airway inflammation. These results confirmed the immunomodulatory potential of *L. plantarum* NCIM8826 *in vivo* and corroborated with our previous findings (6). Similar results were also reported using a recombinant *L. plantarum* NCIMB8826 or *Lactococcus lactis* NZ9800 expressing Bet v 1 (15) and beta-lactoglobulin (32), respectively.

Although a recombinant *L. acidophilus* expressing a mite allergen (Der p 5) used as therapeutic vaccine was first described (30), such recombinant probiotic could not be fully efficient in the treatment of mite allergic patients as only 40–50% of sera from mite allergic patient reacted with Der p 5 (33). As Der p 1 represents one of the most immunodominant mite allergen (33), our study describes for the first time the design and the immunological characterization of a recombinant LAB expressing a major mite allergen.

In conclusion, the present study clearly indicated that *L. plantarum* NCIMB8826 displays a potent pro-Th1 adjuvant effect by its capacity to stimulate mouse dendritic cells through at least a TLR2-, TLR9- and MyD88-dependent mechanism and via MAPK and NF-κB activation. This *L. plantarum* strain producing recombinant Der p 1 represents a promising prophylactic vaccine against house dust mite allergy. Recombinant LAB expressing allergen present many advantages over the traditional subcutaneous and sublingual immunotherapy with allergen extracts: (1) As the allergen is intracellularly produced into bacteria, it is consequently relatively protected from the action of proteases, (2) the good cost-effectiveness of recombinant probiotics by the absence of downstream processing for the allergen purification, (3) the simultaneous presentation of the entire allergen and lactobacilli components, which act as a Th1 adjuvant, at the effector sites of the immune

response. The therapeutic potential of this vaccination system is currently evaluated.

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