

Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy

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Abstract

Recent epidemiological studies and clinical trials suggest a possible role of certain lactic acid bacteria (LAB) strains in the prevention of allergic diseases. In this study, we aimed at evaluating the immunomodulatory potential of two LAB strains, *Lactococcus lactis* and *Lactobacillus plantarum*, for prophylaxis and therapy of allergic immune responses.

Both LAB strains-induced high levels of IL-12 and IFN- γ in naive murine spleen cell cultures. Intranasal co-application with recombinant Bet v 1, the major birch pollen allergen, prior or after allergic sensitization, led to increased levels of allergen-specific IgG2a antibodies and in vitro IFN- γ production, indicating a shift towards Th1 responses. Successful immunomodulation by the mucosal pre-treatment was further demonstrated by suppression of allergen-induced basophil degranulation. We conclude that these LAB strains in combination with an allergen could be promising candidates for mucosal vaccination against type I allergy.

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1. Introduction

The prevalence of type I allergies has constantly increased within recent years [1], leading to the fact that presently up to 30% of children in industrialized countries suffer from symptoms such as allergic rhinoconjunctivitis, allergic asthma or atopic dermatitis [2]. The development of type I allergy, characterized by a dominance of type 2 cytokines (IL-4, IL-5, IL-13) [3], has been linked to several factors, such as genetic predisposition [4] and the nature of the allergenic protein [5]. These factors, however, cannot solely explain the increase in prevalence of atopic diseases. Evidence is accumulating that “western life style”—which is associated with reduced microbial exposure due to high hygienic standards—significantly contributes to the constant increase

of allergies [6–9]. In this context recent studies showed that early childhood exposure to livestock on a farm and ingestion of unpasteurized milk are related with a lower incidence of atopic diseases [10], and furthermore that children living on a farm lose their allergic sensitization more frequently than other children [11].

Lactic acid bacteria (LAB) correspond to a large family of Gram (+) bacteria, of which given genera or species are important members of the indigenous gut microflora in both humans and animals. Dietary LAB strains are also broadly used for preparation of fermented foods and specific strains have been attributed probiotic, i.e. health-promoting properties [12–14]. In the last few years, the possible role of specific LAB strains in the prevention of allergic diseases has become more evident. In particular the relation between the composition of the intestinal flora and the prevalence of allergic diseases has been epidemiologically documented, showing that infants from countries with a high prevalence of allergy—such as Sweden or England—have a

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lower intestinal colonization with certain LAB strains than children from countries where allergic diseases are less prevalent, such as Estonia or Nigeria [15–17]. Moreover, it was recently demonstrated that oral administration of a particular LAB strain (*Lactobacillus rhamnosus* GG) led to reduced atopic dermatitis in children with a positive family history of type I allergy [18], indicating that LAB indeed can exert an anti-allergic effect.

Until now, specific immunotherapy (SIT) [19] is the treatment of choice against type I allergy, performed by repeated injections of increasing doses of allergen extracts. Even though the treatment is successful in many cases there are certain drawbacks, such as frequent injections and long duration, often leading to a low compliance of the patients. Furthermore, aluminium salts which are used as adjuvants in SIT are potent inducers of Th2 responses [20], which might reduce the efficacy of this treatment. Thus, there is increasing interest in improving immunotherapy by the use of Th1 promoting adjuvants [21] and the change to a less invasive route, such as vaccination via the mucosal route [22].

Using a mouse model of type I allergy to birch pollen [23], we have previously tested several mucosal adjuvants for their capacity to modulate an allergic immune response. In this respect, we have demonstrated that immunomodulation can be achieved by the use of mucosal adjuvants such as cholera toxin [23], cholera toxin subunit B [24], or certain bacterial components (CpG-motifs) [25]. In the present study, we tested the capacity of two LAB strains, *Lactococcus lactis* MG1363 (*L. lactis*, a dairy strain) and *Lactobacillus plantarum* NCIMB8826 (*L. plantarum*, a human isolate), to prevent or modulate allergic immune responses. At first, we analyzed the cytokine profile induced by each LAB strain in murine spleen cell cultures. Subsequent intranasal application of these bacteria co-applied with the major birch pollen allergen Bet v 1—prior or after sensitization with the allergen—indicates that such LAB strains are promising candidates for mucosal vaccination against type I allergies.

2. Materials and methods

2.1. Animals

Female, 7-week-old BALB/c mice were obtained from Charles River (Sulzfeld, Germany). Each experimental group consisted of five mice. All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Education, Science and Culture.

2.2. Bacterial strains and growth conditions

Two strains of LAB were used for in vitro stimulation of naive spleen cells of BALB/c mice, as well as for in vivo

applications in the murine model of birch pollen allergy. *L. lactis* MG1363 [26], carrying a plasmid encoding for erythromycin resistance (pTREX 1 [27]) was grown at 30 °C in M17 broth (Difco, Detroit, USA) containing 0.5% (w/v) glucose. *L. plantarum* NCIMB8826 (National Collection of Industrial and Marine Bacteria, Terry Research Station, Aberdeen, Scotland), originally isolated from human saliva, carrying a plasmid encoding for chloramphenicol resistance (pTG 2247 [28]) was grown at 37 °C in MRS broth (Difco). *Escherichia coli* BL 21 (DE3), carrying a plasmid for ampicillin resistance (pMW 172 [29]), was used as a control for in vitro stimulation of naive mouse splenocytes and grown at 37 °C in LB medium (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.5). Antibiotics were used at the following final concentrations: 5 µg erythromycin/ml for *L. lactis*, 10 µg chloramphenicol/ml for *L. plantarum*, and 100 µg ampicillin/ml for *E. coli*.

For in vitro stimulation of naive murine spleen cells, the bacteria were inactivated with 1% phosphate-buffered formalin for 3 h at room temperature, washed twice with sterile phosphate-buffered saline, and stored at –20 °C. Successful inactivation was confirmed by the absence of bacterial growth after plating on selective agar.

For in vivo applications in BALB/c mice, 1:20 dilutions of overnight cultures of *L. lactis* and *L. plantarum* were grown to exponential growth phase. After two washings, the bacteria were adjusted to 10¹¹ colony forming units (CFU)/ml sterile NaCl 0.9% to be used for intranasal application in mice.

2.3. Antigens

Recombinant (r)Bet v 1 was obtained from Biomay GmbH (Vienna, Austria). Birch pollen (Allergon AB, Engelholm, Sweden) was used for preparation of an aqueous extract as previously described [23].

2.4. Animal experiments—treatment protocol (Fig. 1)

Sensitization was performed by three intraperitoneal (i.p.) injections in 10 day intervals with 1 µg (r)Bet v 1 adsorbed to 2 mg Al(OH)₃ (Serva, Heidelberg, Germany) in a total volume of 150 µl. For intranasal application prior to sensitization (prophylaxis) mice were sedated with diethylether, and 10⁹ CFU of *L. lactis* or *L. plantarum* mixed with or without 10 µg of rBet v 1, in a total volume of 30 µl NaCl 0.9%, were intranasally applied eight times (days 0–3 and 7–10) using a finn pipette (Fig. 1). For intranasal treatment after sensitization (therapy), 10⁹ CFU of *L. lactis* or *L. plantarum* were applied with or without 50 µg of rBet v 1 in a total volume of 30 µl NaCl 0.9% three times in 4 day intervals. Control animals were sham treated with sterile NaCl 0.9% before or after sensitization. Mice were killed 7 days after the third intraperitoneal injection (prophylaxis) or 7 days after the last intranasal treatment (therapy).

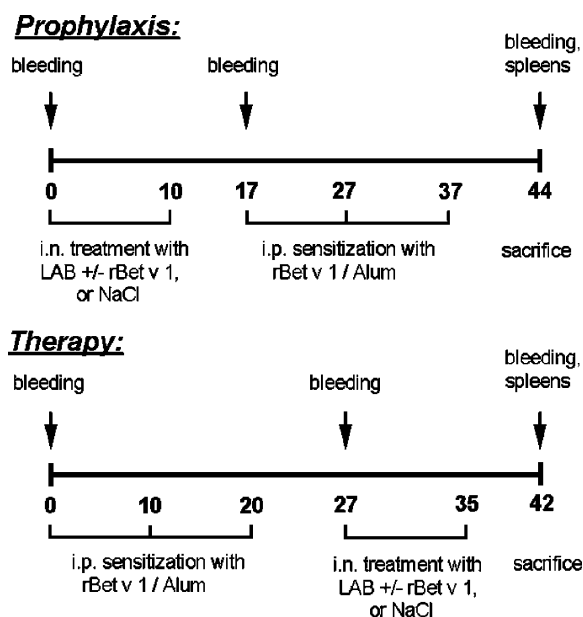


Fig. 1. Experimental design and sampling. Day 0 is defined as the start of the experiment. BALB/c mice were intranasally (i.n.) treated with LAB with or without rBet v 1 prior (prophylaxis) or after (therapy) sensitization with rBet v 1/Al(OH)₃. Blood samples (bleeding) were taken before intraperitoneal (i.p.) sensitization and/or i.n. treatments and before the mice were killed (sacrifice). At sacrifice, spleens were removed for in vitro cytokine assays.

2.5. Sampling

Blood samples were taken by tail bleeding before each sensitization and/or intranasal treatment, and before mice were killed. Sera were prepared for detection of allergen-specific antibodies and basophil release assays, and stored at -20°C until analysis. Spleens were removed under sterile conditions and cell suspensions were prepared as previously described [23]. Briefly, the organs were homogenized, filtered through sterile nylon cell strainers and red blood cells were lysed. The cells were washed and resuspended in complete RPMI 1640 medium (10% fetal calf serum, 0.1 mg/ml gentamicin, 2 mM glutamine and 50 μM 2-mercaptoethanol).

2.6. Determination of allergen-specific antibody levels in sera

Allergen-specific antibody levels were determined by ELISA, as previously described [23]. Microtiter plates (Nunc-Immuno Plate) were coated with rBet v 1 (2 $\mu\text{g}/\text{ml}$). Serum samples were diluted 1/1000 for IgG1, 1/500 for IgG2a and 1/10 for IgE. Rat anti-mouse IgG1, IgG2a, and IgE antibodies (1 $\mu\text{g}/\text{ml}$, Pharmingen, San Diego, CA) were applied and peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Jackson, Immuno Labs Inc., West Grove, PA) were used for detection. Antibody levels were expressed as arbitrary ELISA-units, which were calculated

from a standard curve obtained with a standard serum pool.

2.7. Rat basophil leukemia (RBL) cell degranulation experiments

For determination of the allergenic antibody serum activity, RBL-2H3 cells were plated in 96 well tissue culture plates (4×10^4 cells per well) and passively sensitized by incubation with serum samples of the respective experimental groups in a final dilution of 1:10, 1:30, and 1:100 for 2 h. Unbound antibodies were removed by washing the cell layer twice with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaHPO₄, 5.6 mM D-glucose, 12 mM NaHCO₃, 10 mM HEPES, and 0.1% w/v BSA, pH 7.2) and degranulation was induced by addition of 100 μl of rBet v 1 (0.3 $\mu\text{g}/\text{ml}$ Tyrode's buffer) for 30 min at 37°C . Supernatants were analyzed for β -hexosaminidase content by incubation with 80 μM 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide (Sigma) in citrate buffer (0.1 M, pH 4.5). Reaction was stopped by addition of 100 μl glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at λ_{ex} : 360 nm/ λ_{em} : 465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as percentage of total β -hexosaminidase release after addition of 1% Triton X-100.

2.8. Spleen cell cultures for cytokine analysis

2.8.1. Stimulation of naive splenocytes with LAB

To study the effects of the two LAB strains on the in vitro cytokine production of naive murine spleen cells, 5×10^6 spleen cells were cultured in 48-well flat bottom plates (Costar, Cambridge, MA) at 37°C and 5% CO₂ with or without 5×10^5 , 5×10^6 , and 5×10^7 CFU of formalin inactivated *L. lactis*, *L. plantarum*, or *E. coli*, which was used as a control strain. Supernatants were taken after 24, 48, and 72 h and stored at -20°C until analysis.

2.8.2. Allergen-specific stimulation of splenocytes from treated mice

In vitro cytokine production upon allergen re-stimulation was measured in spleen cell cultures of the different experimental groups. Spleen cells were cultured in 48-well flat bottom plates at a concentration of 5×10^6 cells/500 μl with or without birch pollen (25 μg per well). Supernatants were taken after 48 h for measurement of IL-5 and IFN- γ .

2.8.3. Cytokine analysis

IFN- γ was measured as previously described [30]. All other cytokines were measured with commercially available ELISA kits; IL-4, IL-5, IL-10 (Endogen, Woburn, MA, USA), IL-12+p40 (Biosource International Inc., CA, USA), and IL-13 (R&D Systems, MN, USA), with sensitivities

<5 pg/ml for IL-4 and IL-5, <12 pg/ml for IL-10, <2 pg/ml for IL-12, and <1.5 pg/ml for IL-13.

2.9. Statistics

Allergen-specific antibody levels in mice pre and post-sensitization (prophylaxis and treatment) were compared across groups by Kruskal–Wallis tests. A posteriori comparison of all experimental groups against sensitized controls was done by the rank test analogue to Dunnett's tests. Comparison of cytokine levels induced by the different strains of bacteria was done by two-way analysis of variance after log-transformation of concentrations. The transformation was chosen to reach homogeneity of variances. Linear contrasts for comparison of LAB strains with *E. coli* were performed for each dose. The same analysis was used for evaluation of the RBL cell degranulation experiments. Level of significance was set at 5% for all comparisons.

3. Results

3.1. In vitro cytokine production profile of naive murine spleen cells after LAB stimulation

3.1.1. Type 1 cytokines (Fig. 2)

High amounts of the Th1-promoting cytokine IL-12 were produced after stimulation with increasing numbers of formalin killed *L. plantarum* and *L. lactis* bacteria, in comparison to stimulation with the Gram (–) *E. coli* control strain. IL-12 levels were eight times higher after stimulation with *L. lactis*, and 16 times higher after stimulation with *L. plantarum* compared to the control strain. Consequently, significantly higher levels of IFN- γ were produced by spleen cells

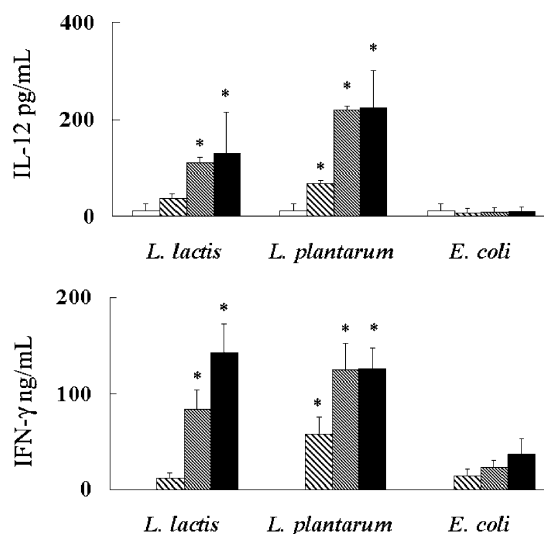


Fig. 2. Type 1 cytokine production of naive murine spleen cells stimulated with bacteria. Splenocytes from naive mice were pooled and cells (5×10^6 per well) were cultured without ($\square$), or with 5×10^5 (diagonal lines), 5×10^6 (cross-hatched), and 5×10^7 (solid black) CFU of formalin inactivated *L. lactis*, *L. plantarum* or *E. coli*, corresponding to 0.1, 1 or 10 CFU per spleen cell in culture. IL-12 and IFN- γ were measured after 48 h of incubation with the bacteria. Bars represent the mean of three independent experiments $\pm$ S.E.M. * is a significant difference between the LAB and the Gram negative *E. coli* control strain ($P \leq 0.05$, linear contrasts after ANOVA).

after stimulation with the LAB compared to stimulation with *E. coli*. Highest concentrations of IL-12 and IFN- γ were detected after 48 h incubation.

3.1.2. Type 2 cytokines (Fig. 3)

In contrast to the high levels of IL-12 and IFN- γ , only low to undetectable levels of type 2 cytokines were measured in spleen cell cultures after stimulation with either *L. lactis* or

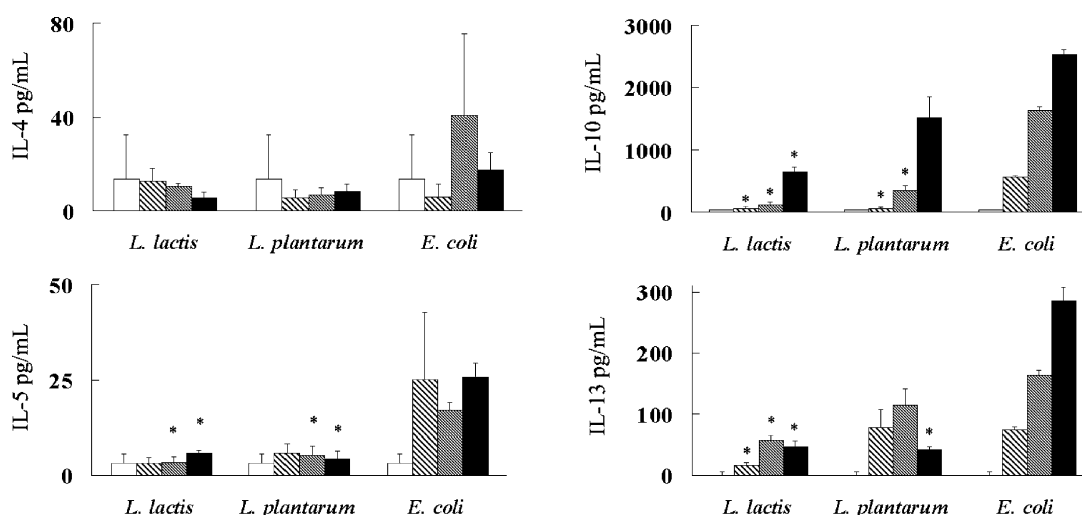


Fig. 3. Type 2 cytokine production of naive murine spleen cells stimulated with bacteria. Splenocytes were pooled from naive mice and cells (5×10^6 per well) were cultured without ($\square$), or with 5×10^5 (diagonal lines), 5×10^6 (cross-hatched), and 5×10^7 (solid black) CFU of formalin-inactivated *L. lactis*, *L. plantarum* or *E. coli*, corresponding to 0.1, 1 or 10 CFU per spleen cell in culture. IL-4 was measured in supernatants after 72 h, all other cytokines after 48 h of incubation with the bacteria. Bars represent the mean of three independent experiments $\pm$ S.E.M. * is a significant difference between the LAB and the Gram negative *E. coli* control strain ($P \leq 0.05$, linear contrasts after ANOVA).

L. plantarum: IL-4, IL-5 and IL-13 levels were markedly lower after stimulation with either of the LAB strains as compared to stimulation with the *E. coli* control strain. IL-10 was induced after stimulation with the highest bacterial concentration of particularly *L. plantarum*, though the amounts were significantly lower than after stimulation with *E. coli*.

3.2. Animal experiments—prophylaxis

3.2.1. Allergen-specific antibody responses (Fig. 4)

Intranasal pre-treatment of BALB/c mice with *L. lactis* or *L. plantarum* mixed with rBet v 1, followed by intraperitoneal sensitization with rBet v 1/Al(OH)₃, induced high and significant levels of allergen-specific IgG2a antibodies. Pre-treatment with the mixture of *L. plantarum* and rBet v 1 induced slightly higher IgG2a levels than the mixture of *L. lactis* and rBet v 1. Mice pre-treated with the LAB strains without rBet v 1, or sham pre-treated with NaCl, produced very low (*L. plantarum*) to undetectable (*L. lactis*, NaCl) levels of this isotype. Although slightly higher IgG1 levels were measured in mice pre-treated with LAB strains mixed with rBet v 1 as compared to the control group, only *L. lactis* reached the level of significance. No significant differences in the allergen-specific IgE antibody levels were measured between the different groups. However, according

to the high IgG2a levels, a 98% reduction of the IgE/IgG2a ratio was calculated for the groups treated with either LAB co-applied with rBet v 1. No allergen-specific IgG1, IgG2a or IgE antibodies were detected subsequent to pre-treatment with the LAB/allergen mixture, but before intraperitoneal sensitization (data not shown).

3.2.2. Rat basophil leukemia (RBL) cell degranulation experiments (Fig. 5)

The allergenic antibody activity of the sera of mice intranasally pre-treated with LAB with or without Bet v 1 was determined in three dilutions (1:10, 1:30 and 1:100) and compared to the sham pre-treated sensitized control group. Sera from groups intranasally pre-treated with *L. lactis* and Bet v 1 as well as *L. plantarum* and Bet v 1 showed a significantly reduced capacity to induce β -hexosaminidase release from rat basophils in comparison to sera from sensitized control mice. In contrast, pre-treatment with *L. lactis* or *L. plantarum* alone did not reduce the allergen-induced basophil degranulation.

3.2.3. Cytokine production profile

IFN- γ levels, induced after in vitro stimulation with birch pollen were markedly increased in spleen cell cultures of mice pre-treated with the mixture of either *L.*

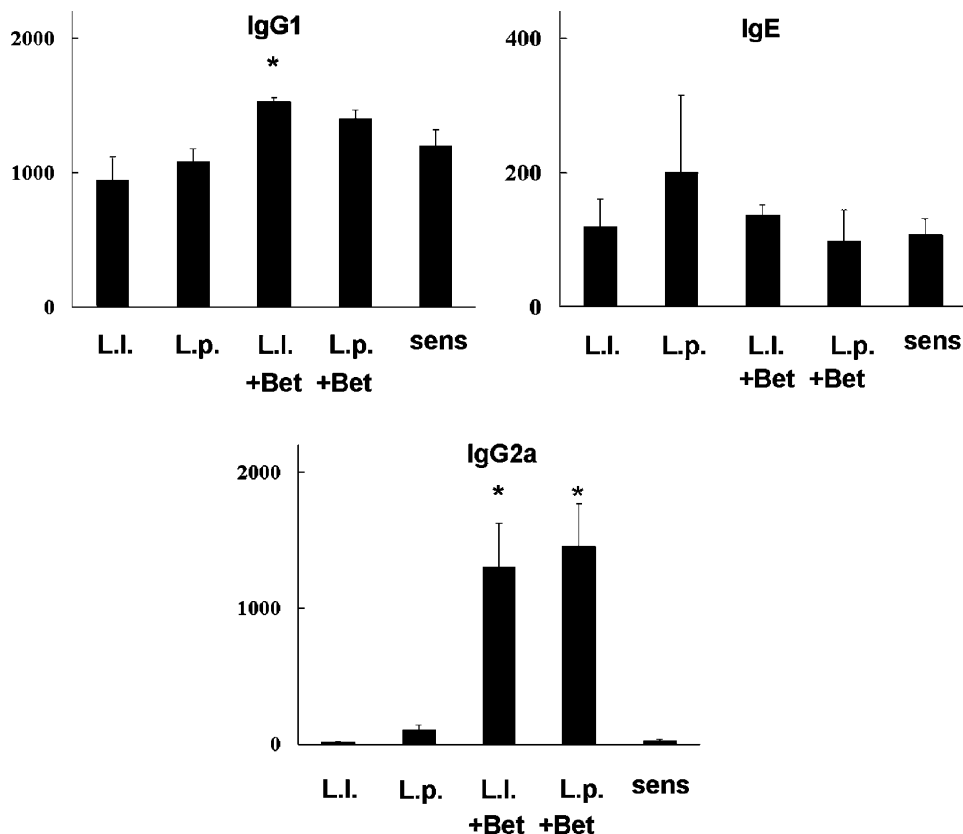


Fig. 4. Allergen-specific antibody levels in sera of sensitized and intranasally pre-treated mice (prophylaxis). Mice were intranasally pre-treated eight times with *L. lactis* (L.I.) or *L. plantarum* (L.p.), with or without rBet v 1 (+Bet), or pre-treated with NaCl (sens). Thereafter, mice were intraperitoneally sensitized with rBet v 1/Al(OH)₃. Antibody levels are reported as mean arbitrary ELISA units $\pm$ S.E.M. * is significantly different from sham pre-treated controls (sens) ($P \leq 0.05$, a posteriori tests after Kruskal–Wallis test).

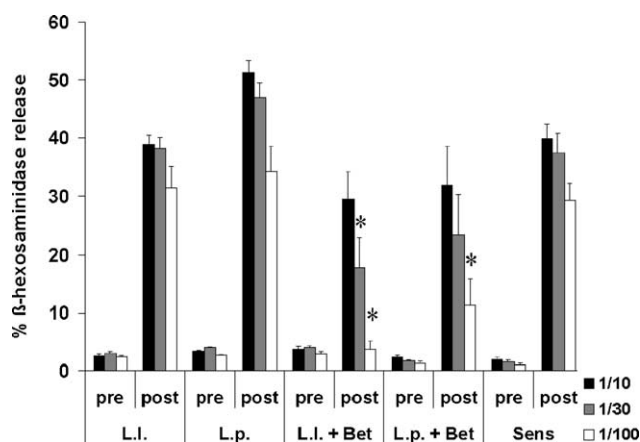


Fig. 5. Rat basophil leukemia (RBL) cell β -hexosaminidase release of sera of sensitized and intranasally pre-treated mice (prophylaxis). RBL-2H3 cells were sensitized with sera of naive mice before treatments (pre) and at sacrifice (post), in dilutions of 1:10, 1:30, and 1:100. Degranulation was induced with rBet v 1, and β -hexosaminidase release is reported as mean percentage of release after degranulation with 1% Triton X-100. Error bars indicate S.E.M.s. * is significantly different from sham pre-treated controls (sens) ($P \leq 0.05$, determined by linear contrasts after ANOVA).

lactis ($P < 0.05$) or *L. plantarum* and rBet v 1, compared to the sham-treated, sensitized control group (Table 1). Pre-treatment with LAB without rBet v 1 was not associated with increased IFN- γ production in vitro. A reduction of IL-5 levels was detected in all groups pre-treated with *L. lactis* or *L. plantarum* with or without rBet v 1.

3.3. Animal experiments—therapy

3.3.1. Allergen-specific antibody responses

Comparing mice treated with LAB or the mixture of LAB and rBet v 1 subsequent to sensitization, a specific increase (3–4-fold) in IgG2a levels was only seen in the groups treated with LAB and rBet v 1 (Table 2). The IgG2a levels in the sham-treated mice were very weak and notably be-

low those of the LAB and rBet v 1 treated mice. In terms of IgG1 and IgE, no changes in the IgG1 antibody levels were detected by the treatments in either of the groups, whereas generally lower IgE levels were measured in all groups after the different treatments.

3.3.2. Rat basophil leukemia (RBL) cell degranulation experiments

The treatment with *L. plantarum* and Bet v 1 led to a 31% reduction, the treatment with *L. lactis* and Bet v 1 to a 25% reduction in the allergen-induced β -hexosaminidase release from RBL cells (Table 2). In all other groups the reduction in the allergen-induced basophil degranulation was less pronounced.

3.3.3. Cytokine production profile

Intranasal treatment with *L. plantarum* administered together with rBet v 1 after sensitization, led to a three-fold increase of IFN- γ production upon allergen re-stimulation in vitro, as compared to sensitized controls, or animals treated with *L. plantarum* alone (Table 3). In the mice treated with *L. lactis* and rBet v 1, a 32% increase in IFN- γ production was observed which however also occurred in the group treated with *L. lactis* alone. IL-5 levels, which were generally rather low, were highest in the group treated with *L. lactis* without rBet v 1.

4. Discussion

Based on the assumption that a lack of counter-regulatory immune responses may influence the development of type I allergy, prophylactic and therapeutic strategies promoting the induction of allergen-specific Th1 responses have been suggested as a promising concept for treatment of Th2-biased hyperresponsiveness. Studies by Riedler et al., showing that early exposure to livestock and farm milk is associated with reduced atopy development, may favour the concept of prophylactically triggering Th1 immune responses [10]. Moreover, the curative effects of Th1 induced responses in established allergy have been seen during successful specific immunotherapy, which is associated with a shift from allergen-specific Th2 to Th1 responses [31–33]. Along these lines, a recent clinical trial indicates that the efficacy of SIT could be enhanced by the use of a Th1 promoting adjuvant [34].

In the present study, we demonstrate that mucosal administration of the two LAB strains—*L. lactis* or *L. plantarum*—applied together with the allergen Bet v 1 prior or after sensitization, induced a shift towards Th1 immune responses along with a reduction of Th2 dependent basophil degranulation. The immunomodulatory capacity of the selected strains in vivo seems to be due to their strong intrinsic capacity to induce the Th1-promoting cytokine IL-12 and consequently the production of IFN- γ from spleen cells, as seen in the in vitro studies. Similarly as described for other LAB strains in

Table 1

Allergen-specific cytokine production in spleen cell cultures of mice intranasally treated prior to sensitization (prophylaxis)

Intranasal treatment prior to sensitization	IFN- γ (pg/ml)	IL-5 (pg/ml) ^a
8 × <i>L. lactis</i>	5139.6 ± 1213.1	52.8
8 × <i>L. plantarum</i>	7357.7 ± 913.9	55.0
8 × <i>L. lactis</i> + rBet v 1	11404.5 ± 774.4*	62.8
8 × <i>L. plantarum</i> + rBet v 1	11144.1 ± 1129.0	82.2
8 × NaCl (sham treatment)	8735.8 ± 584.6	101.2

Cytokines were measured from spleen cell culture supernatants stimulated with birch pollen for 48 h. Mice were treated eight times intranasally with *L. lactis* or *L. plantarum*, with or without 10 μ g of rBet v 1, or treated with NaCl, prior to sensitization with rBet v 1/Al(OH)₃. Data shown are mean values ± S.E.M.

^a Measured from pooled supernatants.

* $P < 0.05$ compared to sham treatment, a posteriori test after Kruskal–Wallis test.

Table 2

Allergen-specific antibody levels in sera and RBL cell degranulation of mice intranasally treated after sensitization (therapy)

Intranasal treatment after sensitization	Antibody levels in ELISA units				RBL degranulation β -hexosaminidase release			
	IgG1		IgG2a		IgE		Post-treatment	Reduction (%)
	Post-sensitization	Post-treatment	Post-sensitization	Post-treatment	Post-sensitization	Post-treatment		
3 × <i>L. lactis</i>	1072.5 ± 183.0	1156.0 ± 167.1	27.9 ± 15.9	27.9 ± 16.2	134.5 ± 32.0	17.3 ± 5.6	31.1 ± 3.5	24.5 ± 2.6
3 × <i>L. plantarum</i>	1332.0 ± 103.0	1361.7 ± 98.5	123.3 ± 71.0	93.8 ± 32.3	278.6 ± 153.8	74.6 ± 26.3	34.2 ± 4.8	27.3 ± 6.8
3 × <i>L. lactis</i> + rBet v 1	995.8 ± 133.3	1378.0 ± 63.9	12.1 ± 7.5	50.0 ± 22.4	87.6 ± 48.5	49.7 ± 19.5	31.6 ± 3.5	23.6 ± 1.9
3 × <i>L. plantarum</i> + rBet v 1	1082.9 ± 115.5	1444.6 ± 58.3	41.3 ± 21.3	120.2 ± 83.9	90.0 ± 35.1	36.4 ± 6.4	38.5 ± 3.7	26.6 ± 2.8
Sham treatment with NaCl	1058.0 ± 151.4	1208.6 ± 83.1	5.9 ± 4.1	15.5 ± 11.6	92.4 ± 29.8	41.9 ± 6.3	30.6 ± 3.4	23.7 ± 3.1

Antibody levels from sera of sensitized mice before (post-sensitization) and after (post-treatment) intranasal treatments are reported as mean arbitrary ELISA units ± S.E.M. Allergen-induced rat basophil leukemia (RBL) cell degranulation is reported as mean percentage ± S.E.M. of maximum β -hexosaminidase release induced with 1% Triton X-100. Mice were treated three times intranasally with *L. lactis* or *L. plantarum*, with or without rBet v 1, or sham-treated with NaCl, after sensitization with rBet v 1/Alum.

mouse models or with human cells [35–38], our data show that in vitro stimulation of naive murine spleen cells with increasing numbers of *L. lactis* or *L. plantarum* bacteria results in the induction of IL-12 and IFN- γ in a dose-dependent manner. The highest IL-12 and IFN- γ concentrations were however induced after stimulation with *L. plantarum*, indicating that this strain might be more effective in modulating Th2 responses in vivo than the *L. lactis* strain. In contrast to *L. lactis* or *L. plantarum*, in vitro stimulation with the Gram (–) *E. coli* control strain also led to a substantial production of Th2 cytokines. In addition to the strong induction of IL-10, which has already been described in human PBMCs [37,39], this *E. coli* control strain also induced higher levels of IL-4, IL-5, and IL-13, as compared to both LAB. In particular IL-13 levels were up to seven times higher after stimulation with the highest number of *E. coli*. The weak capacity of the two LAB strains to induce Th2 cytokines is probably due to their strong induction of IL-12 and IFN- γ , known to inhibit the production or action of IL-4, IL-5, or IL-13 [40–42]. The characterization of the cytokine profile in vitro can therefore give important information for proper strain selection for creating an “anti-allergic” Th1 milieu. The importance of such a characterization has been emphasized by two recent publications, showing that even within the genus of lactobacilli marked differences can be found in the cytokine profile induced by these bacteria [43,44].

Intranasal pre-treatment with the LAB together with the recombinant allergen Bet v 1, led to induction of Th1-biased immune responses, characterized by high allergen-specific IgG2a levels and enhanced IFN- γ production in vitro. Importantly, no increase of allergen-specific IgG2a antibodies was detected, unless the mice were intraperitoneally sensitized with the allergen. In contrast to other studies, showing a reduction of IgG1 or IgE when certain LAB strains were injected or orally applied at the time of sensitization [45–47], we only detected minor changes of these isotypes. However, the fact that the allergen-induced basophil degranulation was significantly reduced after pre-treatment with *L. lactis* or *L. plantarum* mixed with Bet v 1, showed that the shift towards Th1 responses was also associated with a reduction of Th2 dependent reactivities. Thus, besides IgE measurement by ELISA—which might have limitations at low detection levels—the basophil degranulation assay represents a very sensitive method for evaluation of allergen-induced immediate type reactions. At the cellular level, IFN- γ production upon in vitro re-stimulation with birch pollen, was enhanced in the groups pre-treated with *L. lactis* or *L. plantarum* mixed with rBet v 1. The fact that pre-treatment with the bacteria alone did not result in significant allergen-specific immunomodulation (i.e. induction of IgG2a, enhanced IFN- γ levels and reduced basophil degranulation), indicates that the combined application of allergen and bacteria is needed for a specific anti-allergic effect.

Similarly as in the prophylactic approach, only the combined treatment with LAB and the recombinant allergen—subsequent to sensitization—resulted in a substantial

Table 3

Allergen-specific cytokine production in spleen cell cultures of mice intranasally treated after sensitization (therapy)

Intranasal treatment after sensitization	IFN- γ (pg/ml)	IL-5 (pg/ml)
3 $\times$ <i>L. lactis</i>	1278.8	19.3
3 $\times$ <i>L. plantarum</i>	965.7	10.1
3 $\times$ <i>L. lactis</i> + rBet v 1	1145.6	7.9
3 $\times$ <i>L. plantarum</i> + rBet v 1	2372.9	13.9
3 $\times$ NaCl (sham treatment)	866.2	9.7

Cytokines were measured from pooled spleen cell culture supernatants stimulated with birch pollen for 48 h. Mice were treated three times intranasally with *L. lactis* or *L. plantarum*, with or without 50 μ g of rBet v 1, or treated with NaCl, after sensitization with rBet v 1/Al(OH)₃.

increase of allergen-specific IgG2a (Table 2). The highest IgG2a levels were found after treatment with *L. plantarum* and rBet v 1. A general reduction of allergen-specific IgE levels was noted in all groups which might be explained by the short half-life of IgE in the serum [48]. Nevertheless, the reduction of allergen-specific basophil degranulation was most pronounced in those groups treated with *L. plantarum* or *L. lactis* and Bet v 1 (Table 2). At the cellular level (Table 3), the strongest increase in IFN- γ levels was observed in animals treated with *L. plantarum* and rBet v 1. Unlike *L. plantarum*, pre-treatment with *L. lactis* without rBet v 1 also led to enhanced IFN- γ production. However, as IL-5 levels also increased in that group, the IFN- γ /IL-5 ratio did not significantly differ to that of sensitized controls. In contrast, in the *L. lactis* or *L. plantarum* and rBet v 1—treated groups, calculation of the IFN- γ /IL-5 ratio indicated a clear shift towards a Th1 response.

Taken together, our results indicate that prophylactic and therapeutic strategies, based on the induction of counter-regulatory Th1 responses by lactic acid bacteria, are achieved more effectively by a combined mucosal application of the lactic acid bacteria with the specific allergen. The construction of recombinant LAB for local delivery of an allergen might even enhance this protective/immunomodulating capacity against allergies and could represent a useful tool for mucosal vaccination against type I allergy.

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