

Allergy Therapy by Intranasal Administration with Recombinant *Lactococcus lactis* Producing Bovine β -Lactoglobulin

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Key Words

Allergy therapy · β -Lactoglobulin · Th1/Th2 balance ·
Lactococcus lactis

Abstract

Background: In the last years, the use of probiotics such as lactic acid bacteria (LAB) has been proposed as an attractive alternative for the management of allergic diseases. A partial prevention from sensitization to bovine β -lactoglobulin (BLG), one of the major cows' milk allergens, could be achieved in mice after intranasal administration with a recombinant LAB strain, *Lactococcus lactis*, producing BLG (LL-BLG). This study aimed to evaluate the effects of the LL-BLG strain in a therapeutic protocol. **Methods:** Three groups of mice were first orally sensitized to cows' milk and then intranasally administered with either the LL-BLG strain, BLG protein alone or saline solution. Serum samples were collected to analyze BLG-specific IgE, IgG1 and IgG2a, and mice were further intranasally challenged with BLG to elicit a specific allergic reaction. **Results:** Treatment with LL-BLG, but not with BLG alone, contributed to diminish IgG1 production in serum and bronchoalveolar lavage fluids. This was associated with decreased IL-4 production and enhanced IFN- γ production by BLG-reactivated splenocytes, suggesting a switch from Th2- to Th1-immune response. Furthermore, we observed that administration of LL-BLG or LL locally reduced

the allergic reaction induced after intranasal challenge, as evidenced by decreased release of IL-4 in bronchoalveolar lavage fluids. **Conclusion:** These preliminary results demonstrate the efficiency of the intranasal administration of LL-BLG for specific therapy against cows' milk-related allergy.

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Introduction

Cows' milk allergy affects 1.9 to 2.8% of young children in Europe [1], and can develop in adulthood [2]. Patients who are allergic to cows' milk may be sensitized to (i.e. presenting IgE that is specific to) various proteins, but mainly β -lactoglobulin (BLG) and casein [3, 4]. Although most young patients who are allergic to cows' milk outgrow their allergy by 3 years of age, IgE-mediated cows' milk allergy predisposes them to the development of other food allergies and asthma [5]. In susceptible individuals, food allergy results from the inappropriate activation of the Th2-associated immune system, mainly characterized by the production of IL-4 and IL-13 that induce the production of IgE, and of IL-5 which in turn attracts eosinophils. Conversely, the Th1 response is characterized by the induction of IFN- γ and IL-2 production, and stimulation of cellular immunity. Recently, Th17 have also been described and are involved in auto-

immune diseases [6]. Although regulatory T cells are largely involved in suppression of inappropriate immune responses, Th1/Th2 cells have also been demonstrated to regulate their own development via the cytokines produced [7–9]. Thus, reversing the Th2 response by a moderate induction of the Th1 response could represent an attractive strategy for the treatment of allergic disorders. In this context, the immunostimulatory effect of some Gram-positive and nonpathogenic lactic acid bacteria (LAB) strains is of interest, as they were shown to stimulate Th1 cells and inhibit Th2 ones [10–12]. *Lactococcus lactis* is a species of LAB that is widely used in the food industry and has been extensively engineered for the production of therapeutic proteins [13]. *L. lactis* is of particular interest for the mucosal delivery of functional proteins since it is a noninvasive and nonpathogenic food bacterium [14, 15]. In addition, it was recently shown that the use of genetically modified *L. lactis* for mucosal delivery of proteins is a feasible strategy in humans [16].

We have previously engineered a *L. lactis* strain producing BLG (strain hereafter called LL-BLG) and demonstrated that intranasal administration of LL-BLG results in the induction of a BLG-specific immune response in mice [17, 18] and partial protection for further sensitization [19, 20]. The aim of the present study was to evaluate the immunomodulatory effects of LL-BLG strain in a therapeutic protocol, both on ongoing allergen sensitization induced by intragastric administration of cows' milk with cholera toxin, and on the elicitation of the allergic reaction after challenge with BLG.

Material and Methods

Preparation of Live Bacterial Inocula for Immunization

Recombinant LL-BLG strain, named before NZ(pSec:Leiss-BLG) [21], and a *L. lactis* strain transformed with an empty vector (LL), which was used as a negative control, were grown in M17 medium supplemented with 1% glucose (GM17) plus 10 µg/ml of chloramphenicol at 30°C without aeration. To induce the nisin promoter, the 2 strains were grown until OD₆₀₀ = 0.4, and induced with 10 ng/ml nisin for 3–4 h to achieve an OD₆₀₀ of approximately 1.0. Cellular pellets were then harvested by centrifugation (5,000 g, 10 min at 4°C), and washed twice with sterile PBS. The pellets were resuspended in PBS to a final concentration of 5 × 10⁹ CFU in 10 µl and immediately administered to mice.

Mice Sensitization, Treatment with BLG-Producing *L. lactis* and Allergen Challenge

Specific pathogen-free Balb/c mice (6-week-old females; Centre d'Élevage René Janvier, Le Genest Saint-Isle, France) were maintained under normal husbandry conditions, and maintained on a diet deprived of animal proteins. All animal experiments

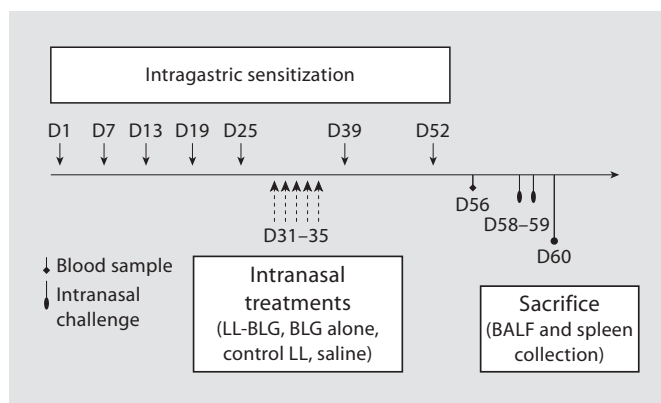


Fig. 1. Experimental protocol. Mice were sensitized by intragastric administration of commercial whole cow's milk with cholera toxin as adjuvant on days 1, 7, 13, 19, 25, 39 and 52. A group of naïve mice were not sensitized ($n = 14$). Mice were then intranasally administered with either LL-BLG or BLG allergen for 5 consecutive days starting on day 31 ($n = 8$ per group). Control mice received either PBS ($n = 9$) or LL ($n = 8$). Allergen challenge was performed in all sensitized and treated mice, and in 8 naïve mice, on days 58 and 59 by intranasal administration of BLG.

were started after 2 weeks of acclimation and performed according to European Community rules on animal care, and with authorization 91-122 of the French Veterinary Services. The sensitization and treatment schedule is detailed in figure 1. Briefly, sensitization was achieved by intragastric administration of commercial whole milk from cows (5 mg of protein/mouse; BioLait; Candia, Lyon, France) adjuvanted with cholera toxin (10 µg/mouse; List Biological Laboratories Inc., Campbell, Calif., USA) on days 1, 7, 13, 19 and 25 as previously reported [22]. Allergic sensitization in all mice was confirmed by assaying BLG-specific IgE concentrations in sera collected on day 31 (data not shown). All mice were then intranasally administered with the different treatments for 5 consecutive days beginning on day 31. During the treatments, the animals were placed under light anesthesia (Isoflurane; Baxter, Deerfield, Ill., USA) and a micropipette was used to administer into 1 nostril 10 µl solutions of either 5 × 10⁹ CFU of LL-BLG ($n = 8$), 5 µg of BLG alone (concentration contained in inoculum of recombinant *L. lactis*, $n = 8$), 5 × 10⁹ CFU of LL ($n = 8$) or PBS ($n = 9$). Mice then received additional cows' milk adjuvanted with cholera toxin on days 39 and 52. A group of mice was not sensitized and not treated (naïve mice, $n = 14$). Allergen challenge was performed in all treated mice and in 8 of the 14 naïve mice ('naïve challenged') on days 58 and 59 by intranasal administration of 10 µg of BLG in 50 µl of PBS, under light anesthesia. This protocol induces a well-characterized allergic reaction, as demonstrated previously [23]. Some naïve mice were not challenged ('naïve', $n = 6$).

Samples and Analysis of the Immune Response after *L. lactis* Treatment

Serum samples were collected on day 56 from the retro-orbital venous plexus. Anti-BLG IgE, IgG1 and IgG2a were deter-

mined on individual sera using immunoassays, as previously described [24]. Twenty-four hours after the final challenge, mice were deeply anesthetized by i.p. injection of 200 µl/mouse of a cocktail of ketamine (15 mg/ml) and xylazine (2 mg/ml; Imalgène 500; Merial, Lyon, France; Rompum 2%; Bayer Pharma, Puteaux, France). The trachea was cannulated and bronchoalveolar lavage fluids (BALF) were collected in sterile saline and kept on ice. Markers of the elicitation of a local allergic reaction were local Th1/Th2 cytokine production and cellular influx, particularly eosinophilia [23]. Total cells in BALF were counted on a Malassez slide after Trypan Blue exclusion, and differential cells counts were performed after cytocentrifugation and staining with May-Grünwald and Giemsa stains (Labo-Moderne, Paris, France). Morphologic characteristics were used for cell differentiation of at least 300 cells/sample, which allowed quantification of eosinophils, lymphocytes, neutrophils and macrophages. Aliquots of the remaining BALF were centrifuged and stored at -80°C until cytokine assays were performed. Th1 and Th2 cytokines [IL-2, IL-4, IL-5, IL-10, IL-12(p70), GM-CSF, IFN-γ and TNF-α] were assayed using BioPlex technology and a mouse Th1/Th2 cytokine kit from BioRad, following the manufacturer's recommendations. All BALF were analyzed individually. BLG-specific IgG1 were also assayed in BALF.

After BALF collections, spleens were removed under sterile conditions to evaluate cytokine production under specific ex vivo restimulation. After spleen dilacerations, red blood cells were first lysed (180 mM NH₄Cl, 17 mM Na₂EDTA) and after several washes the splenocytes were resuspended in RPMI-10 (RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin) and incubated for 60 h at 37°C (5% CO₂) in 96-well culture plates (10⁶ cells/well) in the presence of BLG (20 µg/ml) or filtered cows' milk (25 µg/ml of proteins). Concanavalin A (1 µg/ml) was used as positive control, and saline or OVA (20 µg/ml) as negative controls. Supernatants were then removed and stored at -80°C until cytokine assays were performed (see above).

Statistical Analyses

Data were analyzed by ANOVA, using JMPTM statistical software. Tukey-Kramer's test was used to compare the difference between groups. When multiple comparison was not applied Student's t test was performed. In some experiments comparisons were made using the Kruskal-Wallis test followed by the Dwass-Steel-Christchlow-Fligner multiple comparisons test. $p < 0.05$ was considered statistically significant.

Results

In order to evaluate the effect of BLG-producing *L. lactis* on the ongoing allergic sensitization and on subsequent elicitation of the allergic reaction to a major food allergen, we sensitized mice to cows' milk by intragastric gavages. Once sensitized and before allergen challenge, mice were intranasally administered with LL-BLG, LL, or PBS as a control.

The influence of the treatment on sensitization was evaluated by measuring BLG-specific IgE, IgG1 and IgG2a concentrations in sera and cytokine production on whole milk- or BLG-reactivated splenocytes obtained after sensitization and bacteria administration (fig. 1). As shown in figure 2A, mice treated with LL-BLG produced significantly lower levels of BLG-specific IgG1 than controls (sensitized and PBS-treated mice). The specific IgG1 production in LL-BLG-treated mice was not statistically significant when compared to naïve mice. On the other hand, whereas LL treatment did not induce significant changes when compared to PBS-treated mice, BLG protein evoked a significant increase in specific IgG1 titers. BLG-specific IgE and IgG2a titers were similar in all groups (data not shown). In order to further compare sensitization levels in the different treatment groups, Th1 and Th2-related cytokine secretions were assayed on supernatants of BLG-reactivated splenocytes. As shown in figure 2B, splenocytes from mice treated with LL-BLG demonstrated significantly higher levels of the Th1-related cytokine IFN-γ than control groups (PBS or LL treated), with a concomitant significant decrease in the Th2-related cytokine IL-5 production. Similar results were obtained after reactivation of splenocytes with cows' milk (data not shown). No difference in the other tested cytokines was observed between treated groups (data not shown). These results demonstrate that LL-BLG favored the induction of a specific cows' milk Th1 systemic immune response.

We then measured the local effect of LL-BLG administration on the elicitation of the allergic reaction. Mice sensitized and treated with PBS, BLG, LL or LL-BLG, as well as naïve mice, were intranasally challenged with BLG to elicit an allergic reaction and evoke a local Th2-associated immune response. After allergen challenge, we detected a significant local secretion of IL-4 and IL-5 (fig. 3A), associated with eosinophil influx (fig. 3B) in BALF from PBS-treated mice compared with challenged and unchallenged naïve mice, thus confirming the elicitation of specific Th2-associated reaction by BLG in the Balb/c model of intragastric sensitization to cows' milk. In contrast, treatment with BLG enhances the allergic reaction (fig. 3). No statistically significant difference was found between LL-BLG-treated mice and naïve mice when considering IL-4 release in BALF. These results suggest a discrete but positive role of LL-BLG in the elicitation of the allergic reaction. A modest effect of LL treatment was also observed, as demonstrated by a non-significant IL-4 production in BALF (fig. 3A). On the other hand, we were not able to detect a significant pro-

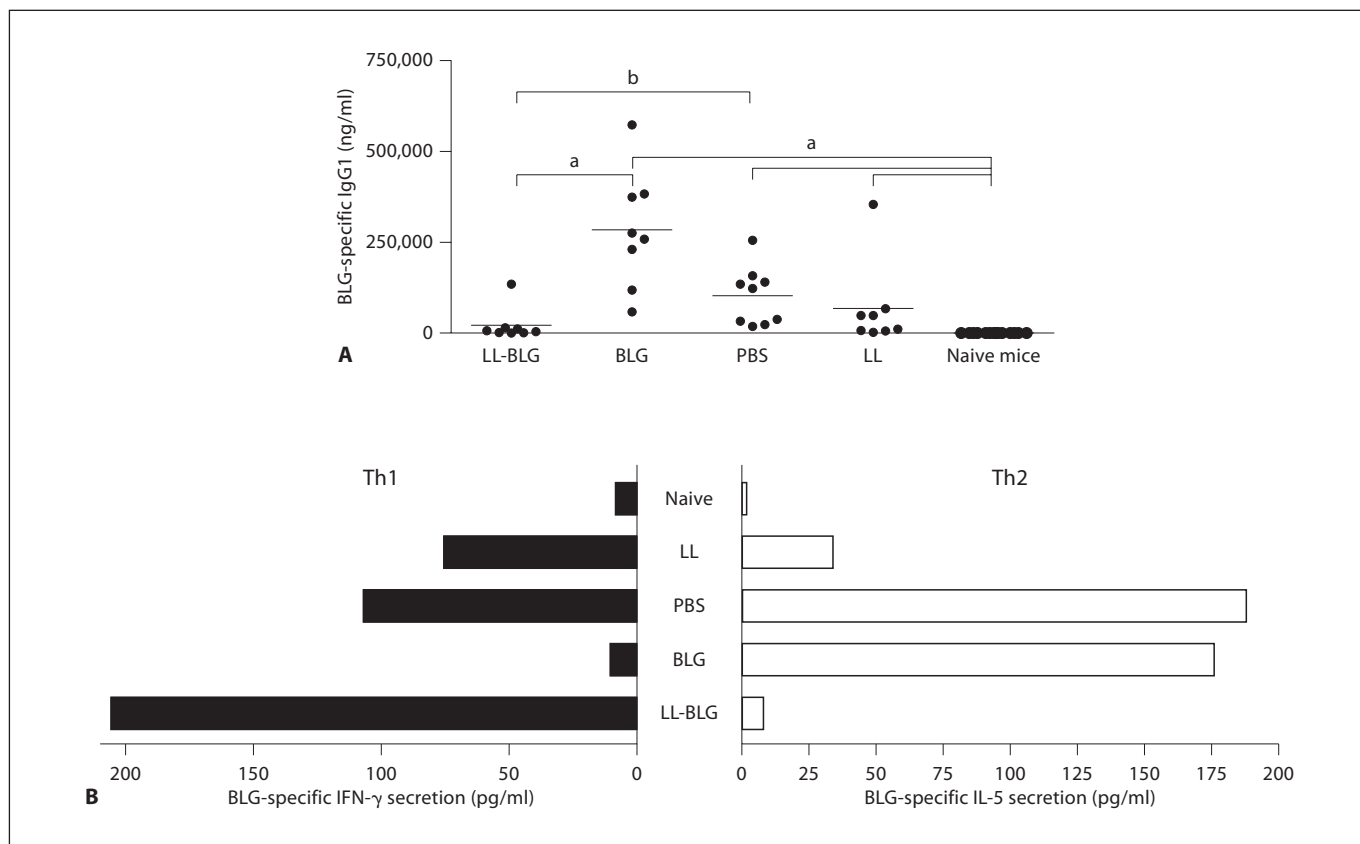


Fig. 2. Effect of intranasal therapy with LL-BLG on systemic immune response. Blood samples of all mice were taken on day 56 and serum anti-BLG IgG1 production was analyzed individually (**A**). After the last nasal challenge, mice were sacrificed and single-cell suspensions of splenocytes were prepared. Cells were cultured

in the presence of BLG, and the levels of secreted IFN- γ (Th1) and IL-5 (Th2) were measured (**B**). Different letters indicate statistically significant differences. ^a $p < 0.05$ using Tukey-Kramer test; ^b $p < 0.05$ using Student's *t* test.

duction of IFN- γ or TNF- α in LL- or LL-BLG-treated mice (data not shown), whereas other cytokine productions were equivalent to those of PBS-treated mice (IL-5; fig. 3A and data not shown). Surprisingly, a higher neutrophil influx was also observed in LL-BLG-treated mice compared to both LL- and PBS-treated mice (fig. 3B).

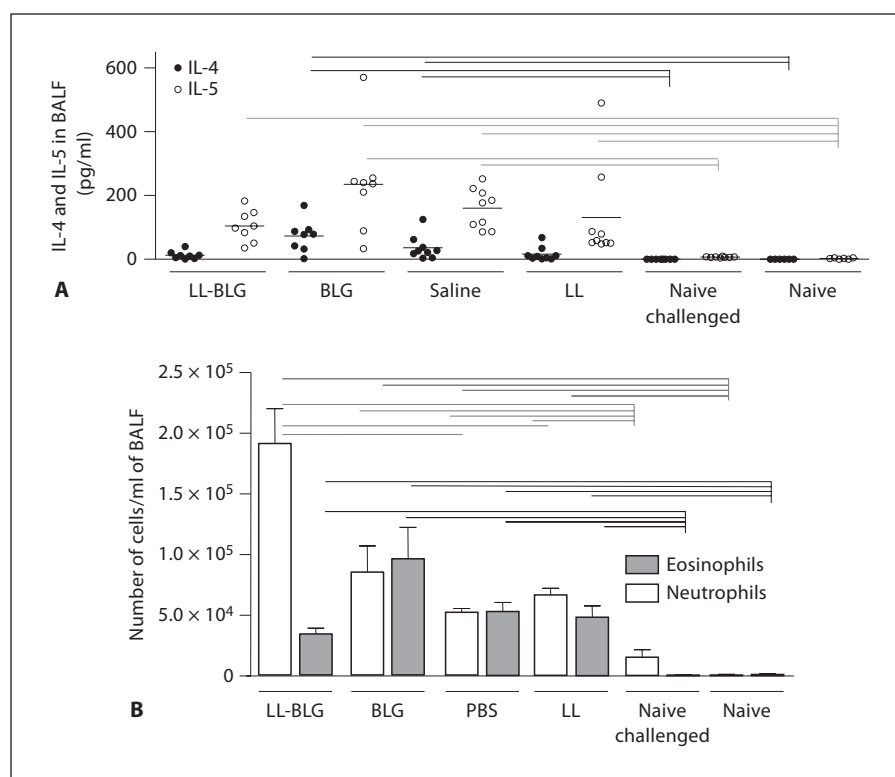
Discussion

We previously demonstrated that oral administration of LL-BLG allows partial prevention of sensitization and induction of a Th1 response to this major cows' milk allergen [19]. In the present study, we examined whether administration of LL-BLG intranasally (a well-known route to elicit a more efficient systemic immune response than the intragastric one [25, 26]), could modulate an

established Th2 response. Balb/c mice were then sensitized to BLG by intragastric administration of cows' milk, and they were further treated with either LL-BLG, LL, BLG or PBS. Firstly, it is worth noting that mice orally sensitized to cows' milk and further treated with PBS demonstrated BLG-specific IgE and IgG1, Th2 cytokine secretion by BLG-reactivated splenocytes, and elicitation of the allergic reaction after intranasal challenge with BLG, as by Th2 cytokine productions and eosinophil influx in BALF, thus demonstrating the relevance of the present model.

Moreover, although serum IgG2a and IgE remain unaffected, our results revealed that LL-BLG treatment significantly decreases BLG-specific IgG1 in sera and BLG-specific IL-5 cytokine production by BLG- and even cows' milk-reactivated splenocytes, whereas IFN- γ secretion was increased. These results suggest that intranasal ad-

Fig. 3. Effect of intranasal therapy with LL-BLG on local immune response. After the last intranasal challenge, mice were anesthetized and BALF were collected. IL-4 and IL-5 (A) and eosinophil and neutrophil influx (B) were then assayed in BALF as described in 'Materials and Methods'. Horizontal lines represent statistically significant differences ($p < 0.05$) using Kruskal-Wallis test followed by Dwass-Steel-Chritchlow-Fligner multiple comparisons.



ministration of LL-BLG, but not LL, can modulate the allergic response by redirecting the Th2 subset towards a mixed systemic Th1/Th2 response, which is in agreement with our previous observations in prophylactic protocols. Modulation of humoral and cellular systemic immune responses should occur through dendritic cell (DC)-loaded recombinant bacteria transportation to systemic lymphoid organs, preferentially via the lymphatics. Accordingly, another LAB, *Lactobacillus casei*, has been shown to be efficiently captured by DCs from intestinal epithelium, allowing a partial maturation of loaded DCs [27]. The simultaneous presentation of BLG and bacterial *L. lactis* components within lymphoid organs via activated DCs should favor the induction of a moderate BLG-specific Th1 response. This may result from the initial modest activation of DC innate immunity by LAB. Indeed, *L. lactis* has been shown to induce a slight but significant production of Th1 cytokines IL-12 and IFN- γ by splenocytes from naïve mice [28]. This then reinforces our strategy of using allergen entrapped in recombinant *L. lactis* to induce a moderate allergen-specific Th1 response to counterbalance the specific Th2 allergic response without inducing a strong adverse Th1 response. Analysis of DCs from draining lymph nodes would fur-

ther address the immunomodulatory mechanisms involved.

Furthermore, we observed that administration of LL-BLG locally reduced IL-4 release in BALF, a marker of the elicitation of allergic reaction induced after intranasal allergen challenge. Surprisingly, the administration of LL partially results in the same effect, suggesting that modulation of airway Th2 reactivity is not allergen specific but may be due to a direct local effect of bacteria, which is the opposite of systemic effects. Although a role for IL-5 and eosinophils in chronic airway changes in the late phase of the allergic reaction is not excluded, IL-4, but not IL-5 and eosinophils, is involved in the acute airway hyper-reactivity in Balb/c mice [29]. IL-4 found in BALF after challenge may result in the intense activation of mast cells after allergen binding to mast cell-bound IgE, as we previously suggested in our BLG-elicitation model [23]. The mast cell-derived IL-4 would participate in the further local activation of Th2 cells. Moreover, it has been demonstrated in Balb/c mice that IL-4 (and IL-13) can act rapidly and directly on the airway-resident cells to induce airway reactivity [30]. Altogether, these data suggest that LL and LL-BLG treatments have a moderate but significant effect on the elicitation of the allergic reaction. On

the other hand, while prophylactic intranasal administration of BLG induced BLG tolerance, intranasal BLG treatment after sensitization further enhances the elicitation of the allergic reaction in the respiratory tract, as hypothesized in our previous study [20]. This further demonstrates the interest of administration of BLG entrapped in recombinant bacteria for our purpose.

Surprisingly, when compared to PBS mice, a higher neutrophil influx was evidenced in LL-BLG- but not in LL-treated mice, but we did not observe significant enhancement of Th1 or inflammation markers such as IFN- γ or TNF- α in corresponding mice. This phenomenon could be explained as being a result from the modest BLG-specific Th1 systemic immune response induced after intranasal LL-BLG treatment. Indeed, a previous study has demonstrated that intranasal administration of recombinant *L. lactis* producing a *Streptococcus pneumoniae* antigen induces a Th1-specific immune response and efficient protection against *S. pneumoniae* challenge [31]. While neutrophils play a role in the containment of pathogen bacteria in the course of the infection via cel-

lular infiltrate [32], their role in airway responsiveness to allergy is largely unknown. A recent study demonstrates that intranasal administration of lipopolysaccharide in young Balb/c mice led to neutrophil influx and transient airway hyperresponsiveness to methacholine, but finally resulted in decreased airway hyperresponsiveness after allergen sensitization and challenge [33]. On the other hand, although efficiently counterbalancing local Th2-mediated pathology, a robust Th1 immune response may lead to intense neutrophil influx and airway hyperreactivity after allergen challenge, as demonstrated in the gene transfer protocol [34]. The impact of Th1-related neutrophil influx observed in the present study would then be studied in chronic asthma protocols, to further confirm its potential beneficial role as a Th2-counterbalancing pathway.

In conclusion, our study provides evidence of an efficient reversal of the Th2-associated immune response by administration of LL-BLG. These promising results represent a step towards the development of new strategies for allergen-specific immunotherapy.

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