

Inhibition of allergen-induced airway inflammation and hyperreactivity by recombinant lactic-acid bacteria

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Abstract

Recombinant lactic-acid bacteria (LAB) are able to inhibit allergen-specific T-cell responses. In this study, we examined whether oral feeding of recombinant LAB was able to suppress allergen-induced airway inflammation and hyperreactivity (AHR) in a murine model. Animals were intraperitoneally sensitized with *Dermatophagoides pteronyssinus* group-5 allergen (Der p 5) and orally treated with recombinant LAB containing a plasmid-encoded Der p 5 gene or placebo on day 7 and day 14 for three days consecutively. Twenty-one days after sensitization, mice underwent inhalational challenging. Der p 5-specific immunological responses including changes to specific immunoglobulin G and E (IgE) levels, the presence of cells in the bronchoalveolar lavage fluid (BALF), and AHR were assessed following this inhalational challenge. We demonstrated that oral feeding of recombinant LAB could significantly decrease the synthesis of Der p 5-specific IgE, and AHR. Furthermore, following such treatment, we also noted that both neutrophils and eosinophils had infiltrated the BALF to a significantly lower extent, when compared to the vehicle-treated group. Neither recombinant allergen nor LAB alone was able to suppress allergen-induced immune responses. Our findings suggest that treatment with recombinant LAB at a low dose can suppress allergen-induced airway allergic inflammation, this providing a basis for developing a novel therapeutic method for allergic airway diseases.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways, currently affecting over 155 million people worldwide and ever increasing in such frequency [1,2]. To the best of our knowledge, allergen immunotherapy is the only currently available treatment that deals with the main cause of allergic disease by modifying or down-regulating the immune response, such a therapeutic modality possibly also altering the natural course of allergic disorders [3,4]. In the past, allergen immunotherapy has been administered mainly by the parenteral injection of allergens but, in recent years, other routes of administration including oral, sublingual, nasal

and bronchial routes have been attempted [5]. Sublingual immunotherapy has been shown to reduce allergic symptoms and/or mediation needs for allergy sufferers in several studies [6–8]. The efficacy of sublingual immunotherapy appears to be similar to that of subcutaneous injection immunotherapy performed upon children, and such success has been ascribed to the use of a higher dosage of allergen and the development of one or more partial “oral tolerance” mechanisms [9,10]. Therefore, the sublingual swallowing method seems to provide the best compromise in terms of efficacy, safety, and patient acceptability [11], although the relatively high cost of such therapy can constitute a serious concern as regards many human therapeutic proteins including crude mite extract [12].

Recombinant allergens featuring an authentic tertiary structure may exhibit the potential to display efficacy and safety profiles similar to those of natural allergens [13–15]. A

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variety of in-vitro, animal and human studies have suggested that yogurt containing live lactic-acid bacterial (LAB) potentiates a variety of immune responses in vitro and may lead to a variety of clinical benefits [16,17]. Regular consumption of yogurt containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* has been shown to improve clinical scores amongst atopic patients featuring rhinitis, and appears to demonstrate a trend toward the increased production of interferon gamma and a decreased level of eosinophilia [18]. Furthermore, some LAB strains have been reported to be able to effectively and safely improve the quality of life of patients suffering from allergic rhinitis [19]. In addition, recombinant LAB has been shown to inhibit allergen-specific T-cell responses [20]. We have constructed a shuttle vector that can express recombinant dust-mite allergen in the *Lactobacillus* and *Streptococcus* bacteria. The allergen can be quite readily released into the culture medium in a stable condition and can be preserved well when stored refrigerated at 4 °C. We therefore investigated whether low-dose oral administration of LAB expressing encoded allergen can effectively down-regulate the synthesis of allergen-specific immunoglobulin E, airway inflammation and hyperreactivity for sensitized animals, compared to animals fed with either LAB or allergens.

2. Materials and methods

2.1. DNA manipulation

Recombinant DNA techniques, in general, were performed as has been described previously [21]. In order to generate promoters for expression of the *Dermatophagoides pteronyssinus* group-5 allergen (Der p 5) gene in *S. thermophilus* and *Lactobacillus acidophilus*, two DNA fragments were acquired by polymerase chain reaction (PCR). The following synthetic oligonucleotide primers were used: primer PUC1233 (complementary to the plasmid pLP3537 sequence from position 4119 to 4096 5'-CTTACGTACGT-CTTGCGC-3'), primer PSD (identical to the plasmid pLP3537 sequence from position 3658 to 3679 5'-AGATCTCCCTCTTTAATTTGGTTATATG-3'). The synthetic oligonucleotide primers described above were numbered according to the scheme proposed in 1991 by Posno et al. [22] and the additional six nucleotides of the 5' ends of primer pSD were designed to yield a *Bgl*III site in the resulting fragment following PCR. Each reaction mixture contained 0.1 µg of template DNA (plasmid pLP3537), 0.25 µg of each primer, 0.2 mM deoxynucleoside triphosphates, 1 U of Taq DNA polymerase, 10 mM Tris-HCl (pH = 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.01% (w/v) gelatin. The amplification protocol comprised 30 cycles of 1 min at 94 °C, 2 min at 55 °C, and 2 min at 72 °C, and was performed in a MiniCycler (MJ Research, Watertown, MA, USA). These reactions yielded a 0.4 kb DNA fragment with the primers pSD and PUC1233. Subsequent to the completion of these reactions, the DNAs were treated with *Hind*III and *Bgl*III,

following which agarose-gel electrophoresis was performed in order to recover the DNA fragments. Each DNA fragment was then ligated with the *Hind*III fragment of plasmid pLP3537 and a 405 bp fragment *Hind*III/*Bgl*III of plasmid pCMVD [23]. The resulting plasmid (pSDDerp5) contained an amplified fragment fused to the cDNA sequence encoding Der p 5.

2.2. Transformation

L. acidophilus (ATCC 4356), *S. thermophilus* and *Escherichia coli* strains were transformed by electroporation using the methods described in 1996 by Walker et al. [24]. The lactic-acid bacteria (and *S. thermophilus*) containing pSDDerp5 were designated as LA-gm and ST-gm, respectively.

2.3. Strains of bacteria

L. acidophilus ATCC 4356 was cultured on MRS broth and agar at 37 °C. *E. coli* strain DH5α was maintained at 37 °C on Luria Bertani (LB) broth and agar. The antibiotics used herein, ampicillin and erythromycin, were purchased from Sigma (The Sigma Chemical Co., St. Louis, MO, USA). For antibiotic selection for *E. coli*, ampicillin and erythromycin were used at concentrations of 50 and 50–100 µg/ml, respectively, whilst for *L. acidophilus*, erythromycin was used at a concentration of 5 µg/ml.

2.4. Animals and study protocol

Female BALB/c mice, aged between six and eight weeks, were obtained from the animal-breeding center of the College of Medicine, National Taiwan University (they originating from the Jackson Laboratory, Bar Harbor, ME, USA), and were divided into four–six groups for each experiment (Table 1). Animals were actively sensitized by intraperitoneal injection of 10 µg of recombinant Der p 5 that had been purified as has been described previously [23]. Mice received LA-gm or ST-gm LAB 10⁹ CFU orally for three days per week for two weeks. There were totally six feedings. Twenty-one days subsequent to the sensitization, animals were exposed to an

Table 1
Characteristics of six experimental groups of test mice and procedures performed for each group

| Group | Number | Mean body weight (gm) | Treatment, total of six doses |
|-------|--------|-----------------------|--|
| A | 12 | 33.3 ± 3.5 | <i>L. acidophilus</i> (LA) 10 ⁹ CFU |
| B | 12 | 32.6 ± 2.2 | <i>L. acidophilus</i> containing plasmid pSDDerp5 (LA-gm), 10 ⁹ CFU |
| C | 12 | 31.9 ± 1.5 | Recombinant Der p 5 (0.1 mg) |
| D | 12 | 31.5 ± 1.4 | Recombinant Der p 5 (0.1 mg) and LA-gm 10 ⁹ CFU |
| E | 12 | 32.2 ± 2.7 | <i>S. thermophilus</i> (ST) 10 ⁹ CFU |
| F | 12 | 33.4 ± 1.3 | ST containing plasmid pSDDerp5 (ST-gm) 10 ⁹ CFU |

aerosol of either 0.1% Der p 5-glutathion S-transferase fusion protein for 20 min or PBS. Eight hours after such inhalational challenge, pulmonary airway resistance was measured for 50 min, and bronchoalveolar lavage fluid (BALF) and serum were collected.

2.5. Determination of Der p 5-specific IgG2a and IgE

The level of Der p 5-specific IgG2a and IgE was determined by ELISA [23]. Protein high-binding plates were coated with 100 μ l of purified Der p 5 diluted in coating buffer (0.1 M NaHCO₃, pH=8.2) to a concentration of 5 mg/ml. Following overnight incubation at 4 °C, plates were washed three times and blocked with 3% (wt/vol) BSA–PBS buffer for 2 h at 25 °C. Sera were used at a 1:100 dilution for IgG measurement and a 1:10 dilution for IgE measurement, both of which were conducted in duplicate. Following overnight incubation at 4 °C, either biotin-conjugated monoclonal rat anti-mouse IgE mAb (PharMinigen, San Diego, CA, USA) or rat anti-mouse IgG2a mAb (PharMingen) diluted in 0.05% gelatin buffer, was added for incubation for an additional hour. Avidin-alkaline phosphatase (1:1000; Sigma) was then added and incubation continued for a further hour at 25 °C, followed by six washes. The colour reaction was developed with the addition of phosphatase substrate *p*-nitrophenyl phosphate disodium (Sigma). Plates were read in a microplate autoreader (Metertech, Taipei, Taiwan) at a wavelength of 405 nm. Readings were referenced to a standard serum sample which had been pooled from six mice which had initially been injected intra-peritoneally (i.p.) with 10 μ g of Der p 5 with 4 mg of aluminium hydroxide and which had been boosted 21 days subsequently with the same dose. The standard serum was calculated as 100 ELISA units/ml.

2.6. Non-invasive method for the determination of airway responsiveness

Using barometric whole-body plethysmography (WBP; Buxco, Troy, NY, USA), the response to inhaled methacholine for conscious, unrestrained mice was measured as has been described previously [25]. Prior to taking the appropriate readings, the box was calibrated with a rapid injection of 1 ml of air into the main plethysmography chamber so as to obtain the 1 mV signal from the WBP. Inspiration and expiration events were recorded by establishing start-inspiration and end-inspiration points, as the box pressure/time curve crossed the zero point. The commencement of an inspiration event was determined by extrapolating from a straight line drawn between two levels of the rising inspiratory phase of the box-pressure signal. The duration of inspiration (T_i) was defined as the time from the commencement of inspiration to the end of inspiration, and the duration of expiration (T_e) as the time from the end of one inspiration event to the commencement of the next. The maximum box-pressure signal occurring during one complete breath in a negative or positive direction was defined as, respectively, the peak inspiratory

pressure (PIP) or peak expiratory pressure (PEP) depending upon what aspect of breathing was being viewed. Recordings of every 10 breaths were extrapolated in order to define the respiratory rate in breaths per minute. The relaxation time (T_r) was defined as the time period that had elapsed until the signal had achieved a value of 36% of the total expiratory pressure signal (area under the box-pressure signal in expiration). This value served as a correlate to the time constant of the decay of the volume signal to a value of 36% of the peak volume during passive expiration. Pause and enhanced pause (P_{enh}) values were defined and calculated by the following equations:

$$\text{pause} = \frac{T_e - T_r}{T_r}$$

$$P_{enh} (\text{enhanced pause}) = \left(\frac{PEP}{PIP} \right) \times \text{pause}$$

As an index of airway responsiveness, all increases in the enhanced pause (P_{enh}) value were determined. Mice were obtained and averaged for 3 min. Aerosized saline, followed by increasing concentrations of methacholine (ranging from 1 to 100 mg/ml), was nebulized for 3 min, following which readings were taken and averaged for 3 min, this occurring subsequent to each nebulization event. Airway responsiveness was expressed as the P_{enh} value per dose of methacholine.

2.7. Assessment of cells in bronchoalveolar lavage fluids (BALF)

Following the measurement of lung-function parameters, mice were cannulated and lavaged with 5 \times 0.5 ml aliquots of 0.9% sterile saline through a polyethylene tube introduced through the tracheostomy. Lavage fluid was collected and then centrifuged (500 \times g for 10 min at 4 °C), and the cell pellet so obtained was resuspended in 0.5 ml of Hank's balanced salt solution. Total cell counts were conducted by adding 10 μ l of the cell suspension to 90 μ l of 0.4% trypan blue following which the cells were counted under a light microscope in a chamber. Differentiated cell counts were made from cytopspin preparations stained by Leu's stain. Cells were identified and differentiated into the following groups: eosinophils, lymphocytes, neutrophils, and macrophages by standard morphological techniques, for which 500 cells needed to be counted under 400-fold magnification and the proportion (percentage) and absolute number of each cell type was estimated.

2.8. Statistical analysis

To assess changes to pulmonary resistance, cytokine concentration, IgE, IgG level, and number of cells in the BALF following Der p 5 challenge, repeated measures for ANOVA were performed in order to compare the differences between the various groups. Following analysis of variance, Duncan

multiple-range testing was used to elaborate upon differences between experimental and control groups. A $p < 0.05$ level was used to indicate statistically significant difference.

3. Results

3.1. Inhibition of allergen-specific IgE response in vivo

Both vehicle-treated and pSDDerp5-treated mice were sensitized intraperitoneally with allergen Der p 5 one week prior to treatment and received an inhalation challenge three weeks subsequent to sensitization. The presence of anti-Der p 5 IgE in the serum three weeks after allergenic challenge was determined by an ELISA. The serum level of Der p 5-specific IgE increased significantly for the vehicle-treated group, whereas pSDDerp5-treated mice revealed more than a 50% inhibition of Der p 5-specific IgE synthesis (Fig. 1). The inhibition of IgE synthesis by recombinant LAB was specific to Der p 5 allergen, because pSDDerp5-treated mice challenged with Der p 2, were shown to be able to produce Der p 2-specific IgE (data not shown). Thus, direct oral feeding of mice with recombinant LAB was able to inhibit in-vivo allergen-specific IgE synthesis efficiently and in an allergen-specific manner. Furthermore, this extent of the suppressive effect upon IgE synthesis by recombinant LAB was more obvious than was the case for oral feeding with either recombinant allergen or untransformed LAB alone (Fig. 1). The differences between transformed species (*Lactobacillus* sp. and *Streptococcus* sp.) did not influence the suppressive efficacy. Combination treatment with recombinant LAB and allergen did not increase the suppressive effect upon the synthesis of Der p 5-specific IgE as compared to animals treated with recombinant LAB only. There appeared to be no inter-group difference as regards Der p 5-specific IgG serum level.

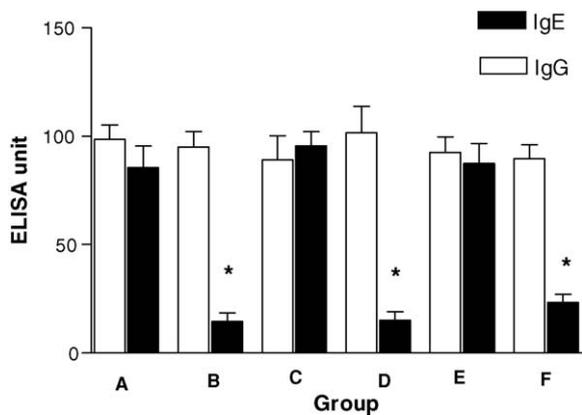


Fig. 1. Der p 5-specific IgG (white bars) and IgE (black bars) levels in serum from Der p 5-sensitized BALB/c mice treated with allergen or recombinant lactic-acid bacteria were determined with ELISA. Values are expressed as mean \pm SEM ($n = 12$ per group). (*) Represents $P < 0.05$ between Group B, Group D or Group F vs., respectively, Group A, Group C and Group E. There is no difference between groups as regards serum IgG level.

3.2. Suppression of airway hyperreactivity (AHR) in vivo

The efficacy and specificity of recombinant LAB for the suppression of Der p 5-specific IgE synthesis prompted us to examine whether oral feeding with recombinant LAB would be able to suppress the allergen-induced AHR. To examine this hypothesis, we measured airway responsiveness in vivo to aerosolized methacholine, 8 h subsequent to the last inhalational challenge for conscious, unrestrained mice. For all mice, a complete dose–response curve for methacholine, over a dose range of from 1 to 100 mg/ml, was constructed. For all groups of mice, no significant inter-group difference was apparent for the basal P_{enh} and saline aerosol-induced P_{enh} values. For mock (LAB or recombinant allergen, Group A, Group C, and Group E)-treated mice, Der p 5 inhalational challenge induced a significant increase in airway responsiveness to methacholine compared with the baseline value. By contrast, treatment with recombinant LAB (LA-gm or ST-gm, Group B, Group D, and Group F) significantly inhibited the AHR value at doses ranging from 10 to 100 mg/ml methacholine (Fig. 2). Thus, Der p 5-induced specific AHR can be abolished by the administration of recombinant LAB following a six-dose treatment regimen. The efficiency did not influence by transformed species. In addition, the inhibition of AHR is specific to pSDDerp5, because there appeared to be no significant difference in AHR for vehicle-treated mice. In addition to the suppression of allergen-induced IgE synthesis, oral feeding of gene-modified LAB

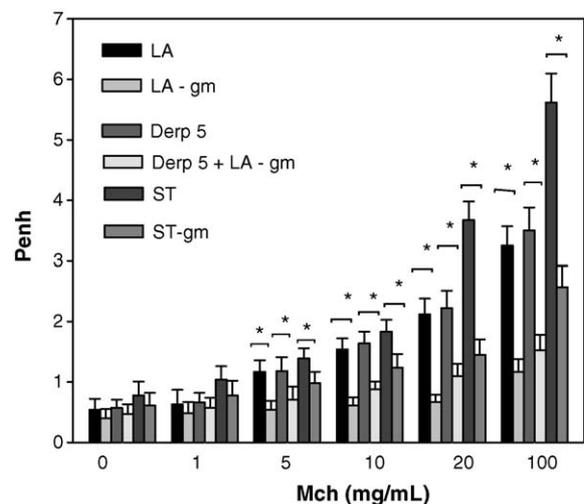


Fig. 2. Animals were intraperitoneally sensitized with Der p 5 on day 1, and treated with *L. acidophilus* (LA), *L. acidophilus* containing plasmid pSD-Derp5 (LA-gm), *S. thermophilus* containing plasmid pSDDerp5 (ST-gm) or recombinant Der p 5, or the combination of both on day 7 and day 14 for three consecutive days. All animals received inhalational challenge with Der p 5 on day 21. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Basal values were measured, followed by measuring the response to nebulized saline and increasing concentrations of methacholine (1–100 mg/ml). Readings for breathing parameters for a period of 3 min subsequent to each nebulization with P_{enh} were determined. Values are expressed as mean \pm SEM ($n = 6$ per group). (*) Indicates $p < 0.05$, as compared to vehicle-treated Der p 5-challenged mice.

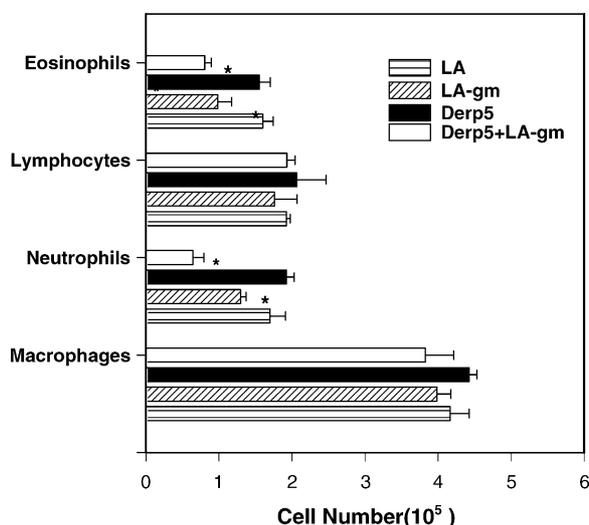


Fig. 3. Numbers of eosinophils, neutrophils, lymphocytes and mononuclear cells in the bronchoalveolar lavage of Der p 5-sensitized mice treated with *L. acidophilus*, *L. acidophilus* containing plasmid pSDDerp5 (LA-gm), recombinant Der p 5 (0.1 mg) or LA-gm with Der p 5. Values are expressed as mean \pm SEM. Per experimental group, six mice were used. (*) Indicates $p < 0.05$, as compared with vehicle-treated Der p 5-challenged mice.

was observed to be able to suppress allergen-induced AHR.

3.3. Bronchoalveolar lavage (BALF)

The number of cells in the bronchoalveolar lavage fluid (BALF) was used as a measure of the relative infiltration of cells into the airways. Significantly low numbers of eosinophils and neutrophils in the BALF of recombinant LAB (Group B and Group D)-treated mice were observed, when compared to vehicle-treated groups (Group A, and Group B; $p < 0.05$). The numbers of macrophages and lymphocytes present in BALF did not differ between the different groups (Fig. 3). Therefore, we conclude that Der p 5 inhalational challenge induces an eosinophilic and neutrophilic cellular infiltration into the BALF. Further, such inflammation was able to be inhibited by treatment with LAB containing plasmid pSDDerp5, but not by treatment with LAB or recombinant allergen only.

4. Discussion

Many investigators have studied the therapeutic and disease-preventive effects of yogurt and lactic-acid bacteria (LAB) for a range of human diseases such as cancer, infection, gastrointestinal disorders, and allergy [26]. Further, earlier studies have demonstrated that LAB cell-wall fractions were able to effectively stimulate the innate immunity system of mice and directly induce interleukin (IL)-12 and interferon- γ production [27]. Furthermore, cytokine secretions are stimulated by the interaction of gram-positive cell components (such as peptidoglycans) with surface

receptors (such as Toll-like receptors, CD14 and others) of peripheral blood mononuclear cells, via the NF- κ B and STAT signaling pathways [28,29]. In addition, it would seem likely that recombinant LAB expressing specific allergen would probably be able to link adaptive and innate immunity for individuals, and also decrease the tolerance dose to allergen. We therefore hypothesize that the cell wall of LAB may be able to elicit some form of an adjuvant effect upon the induction of allergen-specific T cells that antagonize Th2 cell development and associated airway inflammation. Our animal studies have demonstrated that LAB-expressing allergen is able to effectively down-regulate the synthesis of allergen-specific immunoglobulin E (IgE), airway inflammation and hyperreactivity (AHR) for sensitized animals, as compared to animals fed with either LAB or allergens alone. Neither LAB nor allergen at this rather low dosage was able to inhibit the normal allergen-induced immune response. We did observe synergistic effects for the usage of recombinant LAB expressing allergen in the suppression of allergen-induced allergic reaction.

In addition to the production of INF- γ by lymphocytes, we believe that selective suppression of antigen-specific Th2 cells can be achieved by the application of continuous micro-dose oral tolerance [30]. The recombinant LAB usually secreted more than 15 μ g/ml of recombinant allergen after 72 h culture (data not shown). Oral administration of recombinant LAB that could manage to survive intestinal passage could probably result in the secretion of a micro-dose of related allergen and thus result in some level of oral tolerance. These bacteria appear capable of surviving and of being physiologically active at the mucosal surfaces of animals [31]. The oral dose ranges for an allergen required to elicit either a serological or clinical effect appear to be extraordinarily large: perhaps 100–200 times the cumulative dose level necessary to produce the same effect when the allergen is introduced by the subcutaneous route [32]. The use of expensive expression systems such as human or animal cell lines and transgenic animals for such investigation raise concerns regarding cost and safety considerations. Compared to the more traditional subcutaneous and sublingual-swallowing pathways, this new strategy in the treatment of allergic disorder appears to be both cheap and safe. It appears that it will probably not be necessary to purify recombinant allergens for clinical application.

Through the process of genetic engineering, it would appear possible to not only strengthen the therapeutic effects of existing probiotic strains of bacteria, but also to create completely new anti-allergy probiotics. This new strain provides a safe vehicle, and circumvents the short half-life and relative fragility of the therapeutic proteins and produces very cost-effective access to such expensive therapeutics. Recombinant LAB typically is able to survive in the relatively low-pH environment of gastric contents and appears to demonstrate a relatively high tolerance to bile-salt activity. It is, however, also quite clear that the uninhibited spread of genetically modified micro-organisms in the environment is highly undesirable. Such a point relates, essentially, to the absence of

antibiotic selection markers, which could be used to prevent the accumulation of a genetically modified micro-organism in the environment and thus prevent the lateral dissemination of the genetic modification of certain bacteria to other bacteria. These concerns can be addressed through a biological system that is propagated along with the host, i.e. constituting a biological containment system [33].

An evidence-based review validates the successful use of allergen immunotherapy, intranasal immunotherapy and high-dose sublingual immunotherapy as regards ameliorating and preventing the development of allergic rhinitis and asthma [34]. CD4+CD25+ regulatory T cells secreting IL-10, TGF-beta, or both, appear to be important for normal individuals and for patients treated with allergen immunotherapy as regards maintaining or restoring a normal Th1/Th2 balance [35]. It would appear that the precise working mechanisms underlying recombinant LAB use for the treatment of allergen-induced airway inflammation and AHR yet remain to be further elucidated. Our results have demonstrated that this above-mentioned strategy, and subsequent appropriate modifications thereof, will constitute a future modality for appropriate allergen immunotherapy for certain allergic disorders, especially asthma.

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